

# CHARACTERIZATION OF GRANULE-LIKE STRUCTURES IN NON-ENDOCRINE CELLS

## Inauguraldissertation

zur

Erlangung der Würde eines Doktors der Philosophie

vorgelegt der

Philosophisch-Naturwissenschaftlichen Fakultät

der Universität Basel

von

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Basel, 2006



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# ACKNOWLEDGEMENTS

I thank Prof. Martin Spiess for giving me the opportunity to perform my PhD thesis in his lab, but also for project supervision, critical discussions, and for giving good advice.

Special thanks go to Nicole Beuret, Cristina Prescianotto-Baschong, and Gregor Suri for data contributions and the good collaboration.

I would like to thank all members of the Spiess research group, notably Julia Birk, Pascal Crottet, Michael Friberg, Stefan Gander, Veit Goder, Marie Higy, Benjamin Hurschler, Tina Junne-Bieri, Szymon Kobialka, Roman Lutz, Daniel Meyer, Adriana Pagano, and Jonas Rütishauser for help and support as well as for providing a pleasant working atmosphere.

Many thanks also go to Mathis Hafner for mental support during the course of this work.



## SUMMARY

All eukaryotic cells have transport pathways to constitutively secrete proteins. Endocrine, neuroendocrine, and exocrine cells in addition have a regulated secretory pathway that serves the controlled release of hormones, neuropeptides or digestive enzymes. In the trans-Golgi network (TGN) precursors of regulated secretory proteins are segregated and packaged in a concentrated form in secretory granules where they are processed and activated before release in response to an extracellular stimulus by fusion with the plasma membrane. Little is known about the machinery involved in the generation of secretory granules. The current models propose that luminal proteins interact with transmembrane receptors and/or that cargo proteins form insoluble aggregates due to milieu acidification resulting in membrane association and formation of a secretory granule.

We discovered that even in non-endocrine cells the expression of regulated cargo proteins is sufficient to induce granule-like structures. We showed that cargo proteins accumulate in the TGN where they are sorted into membrane vesicles. These structures did not colocalize with organelle markers for the endoplasmic reticulum, the Golgi, the TGN, lysosomes, and endosomes. The helper cargo proteins secretogranin II and chromogranin B were stored intracellularly, and their secretion could be stimulated by addition of a calcium ionophore. Considerable differences were observed in efficiency of induction and in morphology of granule-like structures depending on the expressed cargo protein. Granins were more efficient, capable to sort other cargo proteins, and modulate the size of granule-like structures. Some accessory proteins such as the proprotein convertases PC3 and PC6A but also the IP3-R/ $\text{Ca}^{2+}$  channel sorted into granule-like structures when coexpressed with secretogranin II. The data suggest that secretory granule formation is a self-assembly process which is optimized by an endocrine-specific machinery.

Carboxypeptidase E was proposed to function as a sorting receptor with an unconventional transmembrane anchor similar to PC2 and PC3. The topology of these proteins is particularly important for granule sorting and association with putative interaction partners. A systematic analysis showed that PC3 is a luminal protein suggesting that granule targeting of PCs and CPE is not achieved by direct interaction with cytosolic proteins.



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## ABBREVIATIONS

A1Pi	$\alpha_1$ -protease inhibitor
A1Pi <sup>TS</sup>	Tyrosine sulfation-tagged A1Pi
ACTH	Adrenocorticotrophic hormone
ADP	Adenosine diphosphate
AP-1	Adaptor protein 1
APP	Amyloid precursor protein
ARF1	ADP-ribosylation factor 1
ATP	Adenosine triphosphate
BACE	$\beta$ -site APP cleaving enzyme
BSA	Bovine serum albumin
CCV	Clathrin coated vesicle
CgA	Chromogranin A
CgB	Chromogranin B
CHO	Chinese hamster ovary
CK2	Casein kinase II
COP	Coat protein complex
CPE	Carboxypeptidase E
CSP	Constitutive secretory pathway
EE	Early endosomes
EEA1	Early endosome antigen 1
EM	Electron microscopy
Endo H	Endoglycosidase H
ER	Endoplasmic reticulum
F-actin	Filamentous actin
Fig	Figure
GAP	GTPase activating protein
GEF	Guanine nucleotide exchange factor
GFP	Green fluorescent protein
GTP	Guanosine triphosphate
HEK	Human embryonic kidney
HEPES	N-2-Hydroxyethylpiperazine-N'-2-ethane-sulfonic acid
IF	Immunofluorescence
IP	Immunoprecipitation
IP3-R	Inositol 1,4,5-trisphosphate receptor
ISG	Immature secretory granule
Lamp-1	Lysosome-associated membrane protein 1
M6P	Mannose-6-phosphate
MPR	Mannose-6-phosphate receptor
NPII	Neurophysin II
PACS-1	Phosphofurin acidic cluster-sorting protein

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PBS	Phosphate-buffered saline
PC	Proprotein convertase
PCR	Polymerase chain reaction
POMC	Pro-opiomelanocortin
PP2A	Protein phosphatase 2A
RSP	Regulated secretory pathway
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SgII	Secretogranin II
SG	Secretory granule
SNARE	Soluble <i>N</i> -ethylmaleimide-sensitive factor attachment protein receptor
SNX	Sorting nexin
SRP	Signal recognition particle
SP	Signal peptide
SPC	Subtilisin-like proprotein convertase
TGF- $\beta$	Transforming growth factor $\beta$
TM	Transmembrane
TGN	Trans Golgi network
VAMP	Vesicle associated membrane protein
Vaso	Pro-vasopressin



# INTRODUCTION

## 1. Membrane Traffic

Eukaryotic cells are structured into various compartments or organelles with characteristic functions. These compartments include the endoplasmic reticulum (ER), the Golgi apparatus, endosomes, lysosomes, mitochondria, chloroplasts, peroxisomes, a nucleus and the cytosol. Proteins are synthesized on ribosomes and follow defined transport pathways to reach the compartment of their function. The vast majority of proteins destined to intracellular compartments, the plasma membrane or the cell exterior use the translocation route across or into the ER membrane.

The ER consists of a continuous network of membrane tubules and sacs that extend netlike from the nucleus throughout the cytosol of eukaryotic cells. The ER membranes separate the cytosol from the exoplasmic ER lumen and permit selective transfer of proteins between these two compartments. The ER plays a central role in protein and lipid synthesis. Soluble and transmembrane proteins destined to the ER, Golgi, endosomes, lysosomes, secretory granules, secretory vesicles, and the plasma membrane are initially delivered to the ER lumen.

Integration of membrane proteins into the ER occurs via the Sec61 pore complex. Membrane targeting is achieved via a hydrophobic signal peptide in the primary sequence of nascent proteins (von Heijne, 1990). Upon interaction of the signal peptide with the signal recognition particle (SRP) and its subsequent interaction with the SRP-receptor, the ribosome is docked to the translocon (Keenan *et al.*, 2001). The signal peptide then enters the tunnel, and the nascent polypeptide chain is synthesized into the ER lumen. Polypeptides fold into their three-dimensional conformation and associate with other subunits assisted by chaperones. Translocated segments of proteins can undergo further modifications such as N-linked glycosylation and disulfide bond formation in the ER lumen. In addition, the ER also serves for synthesis of phospholipids, cholesterol and ceramides.

Misfolded proteins cannot exit the ER and are eliminated via ER-associated protein degradation (ERAD) (Meusser *et al.*, 2005). A quality control system within the ER activates the ERAD pathway resulting in dislocation of unfolded proteins and their subsequent degradation by the cytosolic ubiquitin-proteasome system (Nandi *et al.*, 2006). Proteins that meet quality control standards can leave the ER at ribosome-free ER-exit sites for anterograde transport passing through the ER-Golgi intermediate compartment (ERGIC) towards the Golgi (Fig. 1).

The ERGIC is a tubulovesicular membrane cluster which serves as sorting station for anterograde and retrograde traffic (Appenzeller-Herzog and Hauri, 2006). ERGIC membranes receive cargo proteins from ER-exit sites, recycle ER proteins and deliver secretory cargo to the cis-Golgi (Warren and Mellman, 1999).

The Golgi complex is composed of a stack of flattened membrane-enclosed sacs. Proteins enter the complex from the cis-side and are transported through the different compartments towards the trans-side. During transport resident proteins sequentially modify N-linked

carbohydrate side-chains to a complex form and add O-linked oligosaccharides. On the trans-side the Golgi is connected to a tubulovesicular structure referred to as trans-Golgi network (TGN). It is the site of sialylation and sulfation modifications and serves as the cell's main sorting and distribution center. The different types of proteins are sorted and shipped to their final destination. Some are destined for the cell exterior and the plasma membrane while others are transported to intracellular compartments such as endosomes or lysosomes. These sorting processes are crucial for the cell as, for instance, lysosomal enzymes have potential destructive effects and therefore need to be sequestered from the remaining constituents. In addition, the Golgi functions in lipid metabolism notably the synthesis of glycolipids and sphingomyelins.

The endocytic pathway serves for uptake of macromolecules from the cell exterior. Membrane receptors and ligands are taken up by the plasma membrane and transported inward to endosomes, the Golgi and the ER. These transport steps are referred to as retrograde transport. Endocytosed material is initially delivered to endosomes which constitute a major sorting compartment for redistribution to late endosomes, TGN and plasma membrane. Ligand-receptor complexes dissociate due to the reduced pH in endosomes, ligands are delivered to late endosomes and lysosomes for degradation and downregulation of signaling events while receptors are recycled back to the plasma membrane.

## 2. Transport Vesicles

In eukaryotic cells, proteins and membranes are transported along the exocytic and endocytic pathways (Fig. 1) in a strictly controlled and selective manner. Intracellular trafficking is classically mediated by membrane vesicles that vary in size and form from small spherical to larger irregular packages. Transport vesicles mediate the passage of membrane components and soluble molecules from a donor compartment to a target compartment. The molecular mechanisms that control vesicle packaging, budding and fusion have been studied extensively.

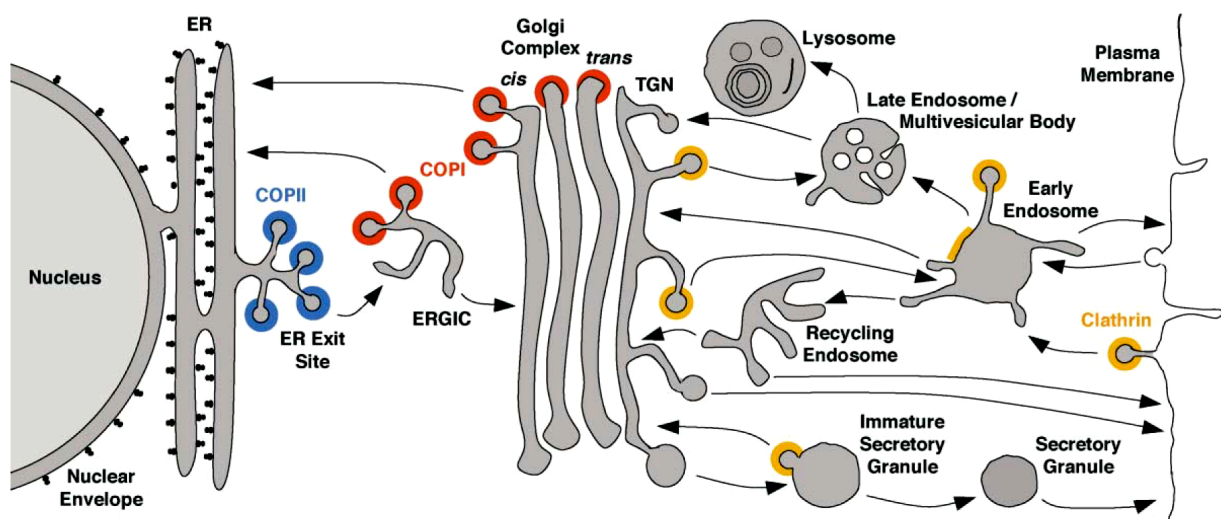


FIG. 1. Illustration of the major membrane traffic pathways using carrier vesicles coated with COPI, COPII, and clathrin (Bonifacino and Glick, 2004).

### 2.1 Coats and Signals

The formation of vesicles requires cytosolic coat proteins that are responsible for cargo protein selection and vesicle budding. Cargo selection is achieved by sorting signals that lie in the primary sequence of cargo proteins. Most transport steps rely on one of the characteristic coat proteins coat protein I (COPI), COPII or clathrin and its partners (Kirchhausen, 2000). COPI vesicles are involved in retrograde traffic from Golgi to ER and in intra-Golgi transport. COPII vesicles mediate anterograde traffic from ER to Golgi. The multiple trafficking routes for clathrin-coated-vesicles (CCVs) are from the Golgi to early endosomes, from the plasma membrane to early endosomes and recycling from early endosomes back to the plasma membrane as well as from early endosomes to the Golgi.

#### 2.1.1 COPI Vesicles

The formation of COPI vesicles starts with the activation of the GTP-binding protein ARF1 (ADP-ribosylation factor 1) by a corresponding guanine nucleotide exchange factors (GEF) at the Golgi membrane. In the active GTP-bound state ARF1 becomes membrane bound via its myristoyl tail which allows subsequent binding of COPI coat proteins. Cargo proteins

containing the dilysine sorting signal K(X)KXX (Cosson *et al.*, 1991) are either bound directly or via the transmembrane KDEL-receptor that in turn binds luminal proteins with a C-terminal KDEL sorting sequence. During coat recruitment the membrane deforms, and when the coat is complete, the vesicle buds off. The carrier vesicle is uncoated in response to ARF1 GTP hydrolysis activated by the GTPase activating protein (GAP) ARFGAP1 (Goldberg, 1999) leading to inactivation of ARF1 and uncoating.

### 2.1.2 COPII Vesicles

Newly synthesized secretory proteins are sorted into COPII-coated vesicles at ribosome-free ER exit sites. COPII vesicles are initiated by membrane recruitment of the cytosolic GTPase Sar1p-GDP upon interaction with the membrane bound GEF Sec12p (Barlowe and Schekman, 1993). Sar1p-GTP then allows the association with the Sec23p-Sec24p complex and cargo proteins/adaptors bearing diphenylalanine or diacidic motifs. In the following step, the scaffolding complex Sec13p-Sec31p binds and deforms the membrane to build a vesicle (Barlowe *et al.*, 1994). GTP hydrolysis of Sar1p activated by the GAP Sec23p leads to uncoating before membrane fusion with a target membrane can occur.

### 2.1.3 Clathrin Coated Vesicles

Clathrin triskelia build a basket-like scaffold that promotes membrane deformation and vesicle budding. For recruitment of clathrin to membranes there exist various adaptors such as the heterotetrameric adaptor proteins (AP-1, AP-2, AP-3, AP-4) or monomeric Golgi-localized  $\gamma$ -ear-containing ADP-ribosylation binding factor protein (GGA) for example. The coat protein clathrin is assembled on adaptor proteins distorting the membranes to form a carrier vesicle. The general sorting sequences for clathrin coated vesicles (CCVs) are tyrosine-based YXX $\Phi$  and dileucine motifs in the cytosolic tails of membrane cargo proteins. The recruitment of clathrin is nucleotide dependent. ARF1 is involved in the recruitment of AP-1 to TGN membranes (Stamnes and Rothman, 1993; Traub *et al.*, 1993) but not in uncoating as it is the case for COPI. The minimal machinery to form CCVs at the TGN consists of myristoylated ARF1, GTP, tyrosine-based sorting signals, specific phosphoinositides, AP-1 and clathrin (Zhu *et al.*, 1998; Zhu *et al.*, 1999). Alternatively, AP-1 was recruited to liposomes in the absence of sorting signals in an ARF1- and cytosol-dependent manner, suggesting the involvement of a cytosolic factor functioning in AP-1 docking (Crottet *et al.*, 2002). AP-1-dependent CCVs further mediate transport from endosomes to the TGN and recycling of receptors to the plasma membrane (Pagano *et al.*, 2004). In a similar way, AP-2 mediates CCV formation at the plasma membrane to permit receptor-mediated endocytosis (Nakatsu and Ohno, 2003) or the internalization of previously exocytosed synaptic vesicle membrane proteins and lipids (Slepnev and De Camilli, 2000). AP-3 was proposed to function in the formation of synaptic vesicles from endosomes of neuroendocrine cells (Faundez *et al.*, 1998). AP-3 and ARF1 were sufficient to reconstitute synaptic vesicle formation from endosomes dependent on ATP and temperature. AP-3 also mediates the delivery of transmembrane glycoproteins from the TGN to lysosomes (Le Borgne *et al.*, 1998). Clathrin disassembly is regulated by the heat shock protein Hsc70 and auxilin in an ATP-dependent manner

(Ungewickell *et al.*, 1995). In addition, various accessory proteins are involved in CCV formation to ensure regulation of the multiple clathrin-dependent sorting events each requiring a specific set of adaptors and regulators. Specific accessory factors involved in cargo selection, interaction with the actin cytoskeleton, vesicle scission and uncoating were reviewed by Lafer (2002).

The mannose-6-phosphate receptor (MPR) is used here as an example to describe the various trafficking routes mediated by CCVs. MPR follows a well-studied trafficking itinerary between Golgi, endosomes and plasma membrane (Fig. 2). The 46 kDa cation-dependent MPR (CD-MPR) and the 300 kDa cation-independent MPR (CI-MPR) share the task of delivering newly synthesized acid hydrolases from the TGN to lysosomes (Kornfeld, 1986; Ghosh *et al.*, 2003). The mannose-6-phosphate modification of an N-linked carbohydrate chain is the sorting signal required for interaction with the MPR. The cytoplasmic domain of the receptor contains a sorting signal for AP-1 and/or GGA binding for subsequent packaging into CCVs destined to early endosomes. During maturation of early to late endosomes, hydrolases are released from the receptor due to acidification of the milieu and delivered to lysosomes via organelle fusion. MPRs do not enter the lysosomal compartment and are recycled from late endosomes back to the TGN regulated by tail-interacting protein of 47 kDa (TIP47) and the GTPase Rab9. Recycling of the CI-MPR from early endosomes to TGN is mediated by PACS-1 and AP1. The CI-MPR is also involved in binding insulin-like growth factor-II (IGF-II) at the cell surface for internalization and delivery to lysosomes, and it mediates the uptake of the serine protease granzyme B involved in apoptosis. MPRs reach the cell surface via recycling endosomes from where they are rapidly internalized in CCVs in an AP2-dependent manner.

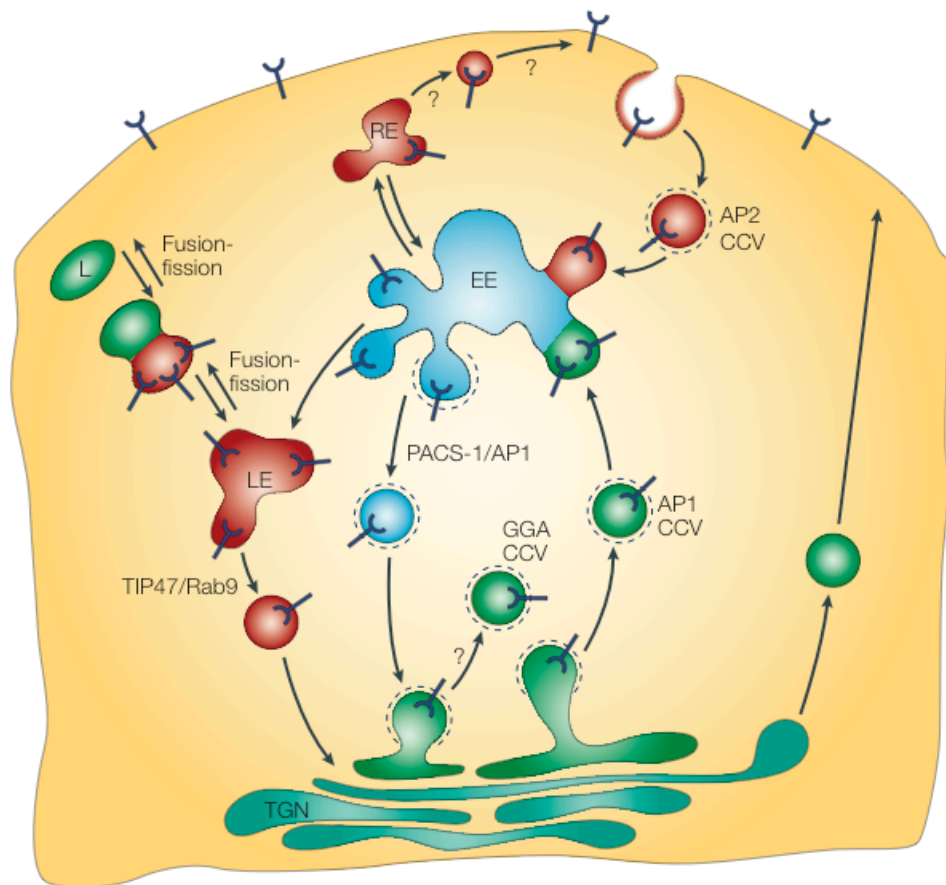


FIG. 2. Overview of the subcellular trafficking itinerary of the MPRs.

MPRs bind acid hydrolases in the TGN, are packaged into AP-1-dependent CCVs and delivered to early endosomes (EE). Hydrolases are delivered to lysosomes while MPRs are recycled back to the TGN from EE by PACS-1-assisted packaging into AP-1-dependent CCVs or from late endosomes (LE) mediated by TIP47 and Rab9 (Ghosh *et al.*, 2003).

#### 2.1.4 Other Transport Vesicles

Secretory granules are involved in regulated secretion of hormones and neuropeptides. However, their formation does not depend on any of the classical coat proteins, and it is not clear if any other coat protein is involved at all. Secretory granule cargo and membrane constituents functionally substitute for classical coat proteins. The machinery underlying the formation of secretory granules is largely unknown. The sorting signals for secretory granules are rather heterogeneous and no classical sorting receptor has been identified. Interestingly, secretory granules differ in size from coated vesicles and have diameter of 100-400 nm while CCVs are typically 100 nm or COP-coated vesicles 60-65 nm thick (Barlowe *et al.*, 1994).

MPR enters immature secretory granules from where it is removed in an AP-1-dependent manner (Klumperman *et al.*, 1998). In a similar way, the TGN/endosomal membrane endoprotease furin (chapter 3.4.3) is excluded from mature secretory granules by CCVs during secretory granule maturation (Fig. 3) (Dittie *et al.*, 1997). This retrieval step requires phosphorylation of the furin acidic cluster by casein kinase II (CK2) for AP-1 binding. The

cycling of furin between the TGN, early endosomes and the plasma membrane is also regulated via phosphorylation by CK2. Interaction with the sorting connector PACS-1 (Wan *et al.*, 1998) and/or AP-1 (Teuchert *et al.*, 1999) or AP-2 clathrin adaptors (Stroh *et al.*, 1999) were shown to be involved in the budding reactions from endosomes, TGN or plasma membrane, respectively. The presence of an acidic cluster within the cytosolic tail of MPR makes it plausible that the mechanism for retrieval is similar to furin removal. AP-4 mediates basolateral sorting of furin in polarized epithelial cells (Simmen *et al.*, 2002), however, AP-4 was not associated with clathrin in electron microscopic studies (Hirst *et al.*, 1999).

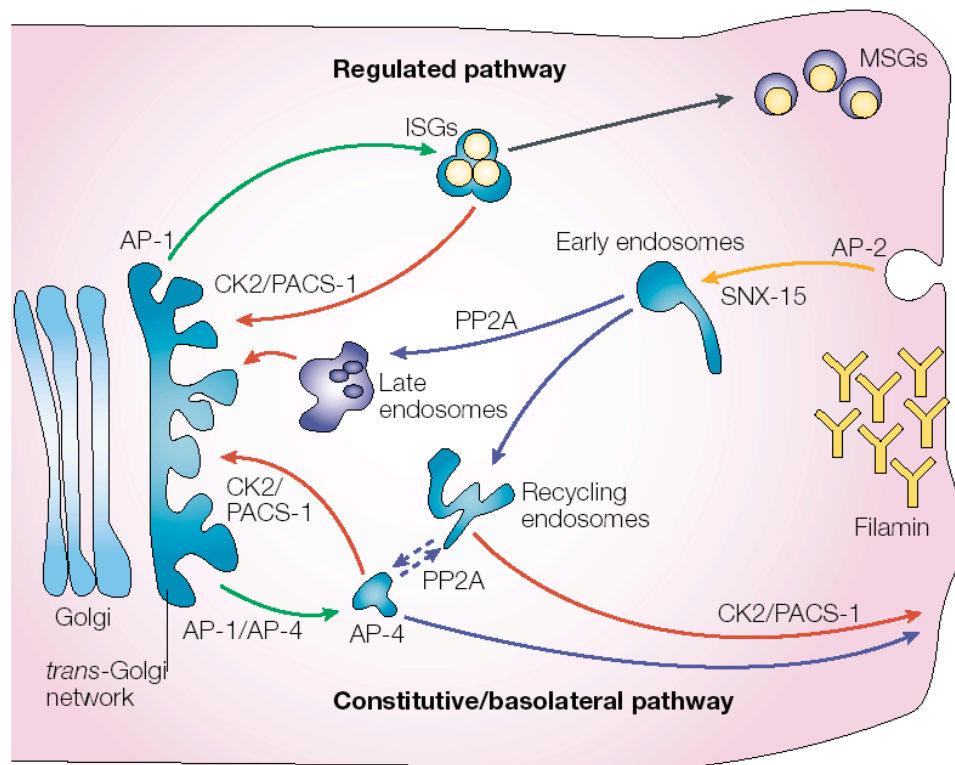


FIG. 3. Scheme depicting the trafficking steps of furin.

In the TGN furin is packaged into AP-1-dependent CCVs for delivery to endosomes. Recycling to the TGN in CCVs is assisted by PACS-1 and AP-1. Basolateral surface targeting is mediated by AP-4, and internalization of cell-surface furin to early endosomes is dependent on AP-2. (Thomas, 2002).

## 2.2 Vesicle Fusion

The final step in the life of a membrane vesicle is its fusion with an acceptor membrane. The targeting and fusion reactions depend on a class of proteins referred to as SNAREs (soluble N-ethylmaleimide-sensitive factor attachment protein receptor). v-SNAREs are found on vesicles and bind to t-SNAREs on a target membrane. Most SNAREs are transmembrane proteins with their functional N-terminal domain facing the cytosol. The main tasks of SNAREs are to promote vesicle fusion and to provide specificity for membrane fusion. The assembly of a four-helix bundle trans-SNARE complex is needed to bring two membranes close enough to each other for spontaneous fusion (Weber *et al.*, 1998). Additional specificity is provided by tethering factors that link opposing membranes prior to SNARE complex

formation. EEA-1 is an example of such a tether and promotes the homotypic fusion of early endosomes (Christoforidis *et al.*, 1999). Tethering factors cooperate with Rab GTPases to promote the initial association of two membranes. Furthermore, vesicle fusion is regulated by a large array of accessory proteins such as synaptotagmin that promotes vesicle fusion in response to  $\text{Ca}^{2+}$  influx (Jahn *et al.*, 2003). N-ethylmaleimide-sensitive fusion (NSF) protein and soluble NSF attachment protein (SNAP) (Weidman *et al.*, 1989) are required for dissociation of the complex and SNARE recycling (Rice and Brunger, 1999).



### 3. Secretory Granules and Regulated Secretion

Organisms must be able to respond quickly to changes in the environment. Hormones and the regulated secretory pathway serve to meet this purpose (Fig. 4). All cells have a constitutive secretory pathway which is considered the default pathway for protein secretion. Some cells are specialized to synthesize and store large amounts of hormones or neuropeptides in order to rapidly discharge them in response to a physiological stimulation. The regulated secretory pathway exists exclusively in endocrine, neuroendocrine and exocrine cells and involves the formation of cytoplasmic storage compartments referred to as secretory granules (SGs) (Fig. 5). Secretory proteins are sorted into SGs where they are concentrated and stored for regulated secretion. The release of regulated secretory proteins requires an extracellular stimulus which triggers the fusion of SGs with the plasma membranes and results in exocytosis of the protein content.

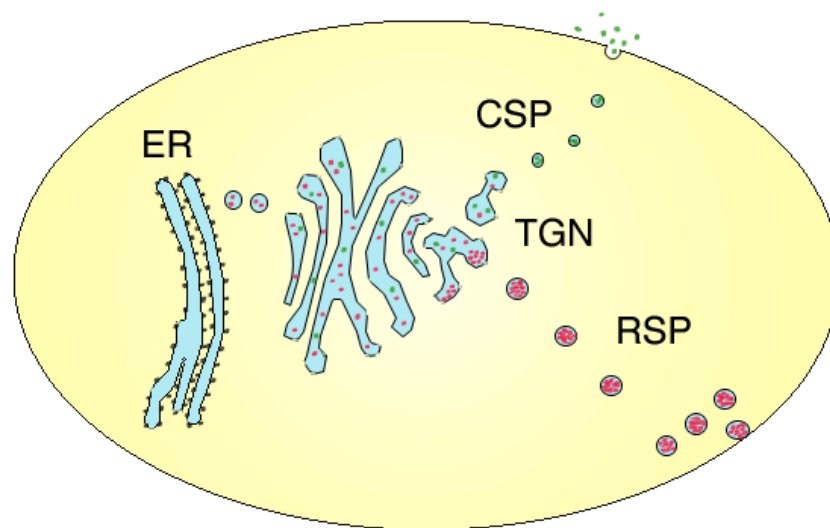


FIG. 4. **Regulated versus constitutive secretion.**

Regulated secretory proteins are segregated in the TGN and sorted into the regulated secretory pathway (RSP) while other proteins destined for the cell exterior follow per default the constitutive secretory pathway (CSP).

#### 3.1 Secretory Granule Formation

Secretory granules are morphologically characterized by their electron-dense core containing hormones or neuropeptides, the main cargo proteins. Other proteins found in the lumen of secretory granules are considered accessory proteins. They include granins that play a role in packaging of the main cargo protein and proprotein convertases which are important for proteolytic processing during granule maturation. These two important classes of proteins are described in chapters 3.4.2 and 3.4.3, respectively.

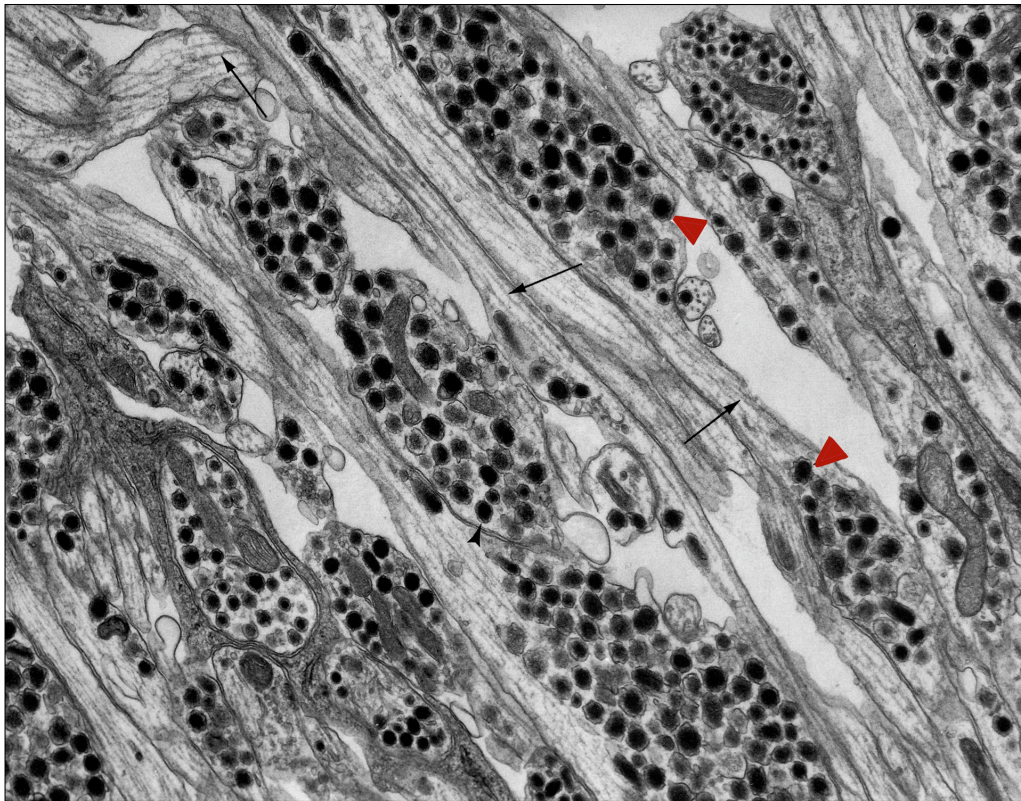


FIG. 5. **Electron microscopic image of the pars nervosa of endocrine glands.**

Secretory granules (red arrowheads) are transported along microtubules (black arrows) to their site of release (28'700x) (Cross and Mercer, 1993).

### **3.1.1 Two Models**

There exist two main models to explain sorting of secretory proteins into the regulated secretory pathway. The sorting-for-entry model suggests a receptor-mediated sorting process (Kelly, 1987) analogous to the M6P-dependent transport of lysosomal enzymes. In a similar way, regulated secretory proteins are specifically bound to a receptor in the nascent secretory granule membrane or to other regulated secretory proteins that are already bound. The aggregated complex is then packaged into a secretory granule by an unknown mechanism independent of classical coatomers. Proteins that fail to interact with a receptor or a membrane bound aggregate will follow the default pathway for constitutive secretion. However, a specific sorting receptor awaits to be identified.

The alternative model, sorting-by-retention, proposes that sorting occurs after formation of an initial immature secretory granule (Arvan and Castle, 1987) and as part of granule maturation. The process is initiated by selective aggregation of regulated cargo proteins in the TGN where low pH conditions and high  $\text{Ca}^{2+}$  concentrations are favorable for aggregation. An immature secretory granule containing contaminating proteins is formed by an unknown mechanism. Captured non-granule molecules are then removed by vesicles that bud from maturing secretory granules via constitutive-like secretion in AP-1-containing

CCVs (Klumperman *et al.*, 1998). The aggregated specific secretory proteins are retained in the maturing secretory granule and become even more condensed during this process.

### 3.1.2 Sorting Signals

For sorting into the regulated secretory pathway no general consensus motif has been identified. Amino acid sequence and secondary structure analysis of 15 prohormones and propeptides revealed a degenerate Ser-Leu-Leu motif on one side of an amphipathic helix which was suggested to serve as a targeting sequence (Kizer and Tropsha, 1991). However, the available data on identified sorting signals are very heterogeneous and suggest that the sorting information does not lie in a conserved primary sequence.

For instance, a C-terminally truncated and constitutively secreted form of furin was redirected to the regulated secretory pathway of AtT-20 cells when fused to the 48 amino acid C-terminal end of the prohormone convertase PC2 (Creemers *et al.*, 1996). Fusion of the C-terminal 51 amino acids of the processing enzyme carboxypeptidase E (CPE) to albumin resulted in membrane association and sorting of this chimeric protein to the regulated secretory pathway of AtT-20 cells (Mitra *et al.*, 1994). The C-terminal segments of CPE and PC2 form amphipathic helices which are thought to be responsible for membrane interaction and thereby direct sorting into the regulated secretory pathway (Fricker *et al.*, 1990). The presence of a sorting signal in the C-terminal 38 amino acids of the prohormone convertase PC6A was shown by inefficient entry into the regulated secretory pathway of a deletion mutant. The isoform PC6B does not contain this C-terminal segment and does not enter the regulated secretory pathway (De Bie *et al.*, 1996) further underlining the presence of a sorting signal in the C-terminus of PC6A. Disruption of a disulfide bond at the N-terminus of POMC resulted in its constitutive secretion in Neuro2a cells suggesting that an amphipathic loop structure stabilized by a disulfide bridge contains the sorting information for targeting POMC to the regulated secretory pathway (Cool *et al.*, 1995). Similarly, the reduction of the single disulfide loop at the N-terminus of CgB with dithiothreitol resulted in its constitutive secretion in PC12 cells (Chanat *et al.*, 1993). The regulated secretory proteins POMC, CgA, CgB, prosomatostatin, and proenkephalin contain a hydrophobic peak within their N-terminal 40 amino acids that overlaps with a predicted  $\alpha$ -helix (Gorr and Darling, 1995).

It was suggested that many regulated secretory proteins exist in a soluble and in a membrane-bound form. The tight association with the granule membranes may be achieved via these amphipathic protein segments in a pH-dependent manner. Soluble proteins cluster around the membrane associated populations and form multimers that eventually lead to the creation of a specialized sub-domain in the TGN membrane from which immature secretory granules can form. It has been suggested that lipid rafts contribute to the budding of immature secretory granules. Lipid rafts are membrane subdomains enriched in cholesterol and sphingolipids forming a dynamic assembly of liquid-ordered phases in the lipid bilayer (Simons and Toomre, 2000). An important property of lipid rafts is their preferential interaction with selected proteins allowing to include or exclude proteins to variable extents.

## 3.2 Granule Maturation

During maturation of immature secretory granules, homotypic fusion occurs and the assembly of a dense core is promoted by mild acidification of the intraluminal environment and the presence of calcium ions. In this process, regulated secretory proteins are greatly condensed to as much as 200-fold the concentration in the Golgi. At the same time, cargo proteins undergo proteolytic processing by proprotein convertases for precursor activation. Processing also has an influence on cargo retention since unprocessed prohormones are removed together with contaminating non-granule proteins (Kuliawat *et al.*, 2000). Protein contaminants that do not belong into the regulated secretory pathway are removed via CCVs in so-called constitutive-like secretion (Klumperman *et al.*, 1998). It has been proposed that regulated secretory proteins are retained in secretory granules through association with phospholipids of the granule membrane in particular with phosphatidylcholines, lysophosphatidylcholines and sphingomyelins (Laine and Lebel, 1999). Outward transport along microtubules occurs concomitantly with all these maturation processes including condensation, processing reactions, removal of contaminants and remodeling of the granule membrane. Mature secretory granules are stored in the cell periphery, near the plasma membrane, for regulated exocytosis.

## 3.3 Exocytosis of Secretory Granules

The release of secretory granules requires several sequential stages such as tethering and docking at the release sites, priming (conversion to a fully releasable state), triggered membrane fusion, release of vesicle contents and retrieval of membranes. Mature secretory granules stay either in the proximity of the plasma membrane or at some distance from it. In PC12 cells, mature secretory granules are frequently adjacent or directly docked to the plasma membrane even before stimulation. However, in other neuroendocrine cells, as well as in neurons, they remain at some distance and move to the plasma membrane only when the cell is stimulated. Rab27A and MyRIP (Myosin VIIA and Rab interacting protein) were proposed to bridge SGs to F-actin and to control short-range movements near the plasma membrane and towards release sites (Desnos *et al.*, 2003). Overexpression of Rab27A or MyRIP inhibited secretion and restricted the movement of secretory granules within the actin cortex. The actin network forms a mesh smaller than a granule diameter, and therefore the motion of secretory granules within the actin cortex depends on remodeling of the actin network to promote or restrict the mobility of secretory granules and their access to the plasma membrane. Granuphilin (a member of the synaptotagmin-like protein family) was proposed as the principal docking factor for insulin granules and to molecularly link insulin granules to the fusion machinery (Gomi *et al.*, 2005). Granuphilin is a Rab27A effector and directly binds to the plasma membrane anchored SNARE syntaxin 1 (Torii *et al.*, 2002) and to Munc18-1 (Coppola *et al.*, 2002) in the closed, inactive conformation using syntaxin 1 or the syntaxin 1-Munc18-1 complex as a recognition platform at the plasma membrane.

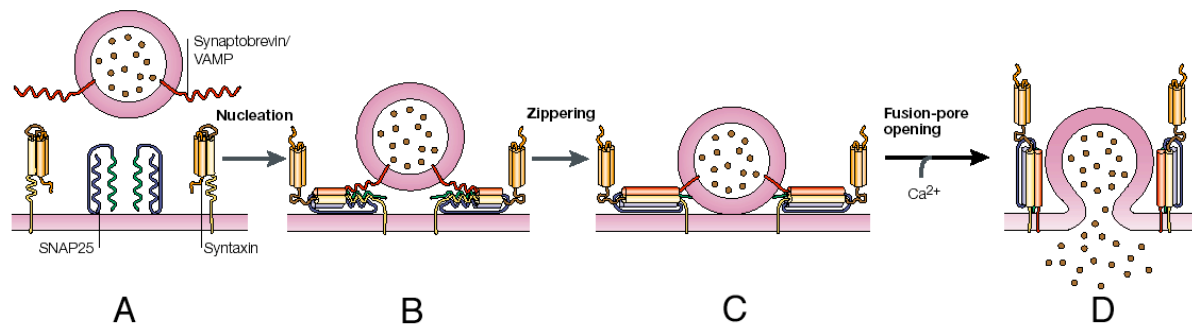


FIG. 6. **SNARE complex assembly in synaptic exocytosis.**

Syntaxin exists in a closed conformation (A) which needs to open for nucleation of the fusion complex (B). Syntaxin, SNAP25 and VAMP form a four-helix bundle which brings the vesicle close to the membrane (C). Elevated intracellular  $\text{Ca}^{2+}$  levels in response to an extracellular stimulus cause opening of the fusion pore (D) (adapted from Rizo and Sudhof, 2002).

Molecularly docked granules are fusion-incompetent and need to be primed for fusion analogous to secretory vesicles. In the priming reaction, the docking complex is resolved and syntaxin 1 changes to an open conformation which allows to form a four-helix bundle complex with the SNARE proteins SNAP25 and synaptobrevin/VAMP, bringing secretory vesicle and plasma membrane toward each other (Rizo and Sudhof, 2002) (Fig. 6). Interestingly, newer secretory granules are preferentially exocytosed (Solimena and Gerdes, 2003). The youngest granules are localized in close proximity to the plasma membrane while the older ones are to the interior of the cell. The fusion pore opens in response to an extracellular stimulus which is often a hormone that binds to a receptor at the cell surface resulting in a transient increase in free  $\text{Ca}^{2+}$  concentration. After fusion, protein complexes are disassembled and recycled together with excessive membranes.

### 3.4 Granule Constituents

The lumen of secretory granules contains hormones or neuropeptides, proteinases, matrix proteins, calcium ions and protons (Apps, 1997). Many luminal constituents are membrane-associated. Integral membrane proteins include proton pumps, channels, transporters, cytochrome  $b_{561}$  and proteins functioning in trafficking. The granule membrane lipids mainly include cholesterol, phosphatidylethanolamine, (lyso)phosphatidylcholine, and sphingomyelin (LeBel and Beattie, 1984; Dhanvantari and Loh, 2000).

#### 3.4.1 Hormones and Neuropeptides

Hormones and neuropeptides are the main cargo proteins of secretory granules. They are synthesized and stored in large amounts in neuroendocrine cells to be available for instant release. Many peptide hormones and neuropeptides are synthesized as large inactive precursors which are cleaved into their final active form only after sequestration in secretory granules. The precursors or parts of them assist folding or assembly of hormone subunits and serve for efficient transport through the secretory pathway. In addition, some precursors can give rise to different hormones depending on the available processing enzymes.

### *Insulin*

Insulin is the prototype of polypeptide hormones (Steiner *et al.*, 1969). Proinsulin is synthesized in pancreatic  $\beta$ -islet cells and plays an important role in regulating blood glucose levels. Preproinsulin consists of an N-terminal signal peptide followed by the B-chain, a connecting peptide, and the A-chain. Proteolytic processing and disulfide bridge formation between A- and B-peptides leads to the generation of active insulin which is stored in secretory granules of  $\beta$ -cells. In response to high glucose levels insulin is exocytosed and released into the blood stream to induce glucose uptake in target cells.

### *Vasopressin and Oxytocin*

The physiological role of vasopressin lies in adjusting the water balance in the kidney. Vasopressin is synthesized in vasopressinergic neurons of the hypothalamus and reaches kidney cells via the endocrine secretion system. The inactive prepro-vasopressin consists of an N-terminal signal peptide, followed by the vasopressin nonapeptide, the carrier protein neurophysin II and a glycopeptide of unknown function. The signal peptide is cleaved after translocation into the ER; neurophysin II and the glycopeptide are cleaved by specific convertases in secretory granules to render the biologically active vasopressin nonapeptide. Oxytocin differs only by two amino acids from the vasopressin nonapeptide. Its precursor contains the carrier protein neurophysin I but no glycopeptide. Oxytocin causes uterus contraction and functions in stimulating milk production in the mammary gland.

### *Pro-opiomelanocortin (POMC)*

POMC is a precursor for various peptides which are involved in diverse biological functions (Raffin-Sanson *et al.*, 2003). In a tissue-specific manner it undergoes extensive post-translational processing mainly by PC2 and PC3. In corticotroph cells of the anterior pituitary POMC is processed to NT (N-terminal peptide), JP (joining peptide), ACTH (adrenocorticotrophin),  $\beta$ - and  $\gamma$ -LPH (lipotrophin), and  $\beta$ -endorphin.  $\alpha$ -,  $\beta$ - and  $\gamma$ -MSH (melanocyte-stimulating hormone), CLIP (corticotrophin-like intermediate lobe peptide), and  $\beta$ -endorphin<sub>1-27</sub> are produced in the intermediate lobe and the hypothalamus. Production of ACTH and  $\alpha$ -MSH has been observed in skin cells where it influences skin pigmentation. Chemical modifications further contribute to biological activity and diversity of POMC-derived peptides.

## **3.4.2 Granins**

Granins are a unique group of acidic, soluble secretory proteins isolated from secretory granules of distinct endocrine cells (Huttner *et al.*, 1991; Taupenot *et al.*, 2003). They have been proposed to act as helper proteins for sorting and packaging of peptide hormones and neuropeptides into secretory granules (Rosa *et al.*, 1985). They are thought to act as assembly factors which coaggregate with other secretory proteins and enable their condensation.

Chromogranin A (CgA) was the first to be discovered and has been studied extensively. It has been considered as the index member of this family. The other six members are chromo-



granin B (CgB), secretogranin II (SgII or CgC), secretogranin III (SgIII or 1B1075), secretogranin IV (SgIV or HISL-19), secretogranin V (SgV or 7B2), and secretogranin VI (SgVI or NESP55). The granins generally have little sequence homology, apart from the N-termini and the disulfide-bonded C-termini of CgA and CgB. They are ubiquitously distributed throughout endocrine and neuronal tissues, and they are cosecreted with tissue-specific hormones and neuropeptides. They are very hydrophilic with a high number of charged amino acids and acidic isoelectric points (Table I). Granins bind calcium and aggregate *in vitro* at low pH in the presence of calcium ions. These properties have been implicated in the mechanism responsible for sorting to secretory granules. It has been reported that CgA and CgB have granulogenic effects and are able to induce *de novo* secretory granule biogenesis in non-endocrine cells (Huh *et al.*, 2003). It has even been suggested that CgA acts as a main on/off switch that triggers or blocks secretory granule biogenesis (Kim *et al.*, 2001).

Property	CgA	CgB	SgII	SgIII	SgIV	SgV	SgVI
Chromosome localization	14 (human), 21 (bovine), 6 (rat), 12 (mouse)	20 (human), 3 (rat) 2 (mouse)	2 (human), 9 (rat), 1 (mouse)	2 (mouse)	ND	15 (human), 2 (mouse)	20 (human)
Amino acids (without signal peptide)	431-445	626-657	559-586	449-507	ND	185	241
Molecular mass (kDa),							
Calculated	49-52	48-52	67.5	51-57	ND	21	27.5
Apparent	74-80	100-120	86	57	35	23	55
Acidic residues (%)	25	24	20	19	ND	16	21
Isoelectric point (pI)	4.5-5.0	5.1-5.2	5.0	5.1	5.6	5.2	4.4-5.2
Multibasic sites	8-10	15-18	9	6-10	ND	3	5
Disulfide-bonded loop	Yes	Yes	No	No	ND	No	No
Calcium binding	Yes	Yes	Yes	ND	ND	Yes	ND
Thermostability	Yes	Yes	Yes	ND	ND	Yes	Yes
Phosphorylation	Yes	Yes	Yes	ND	ND	Yes	Yes
Sulfation	Yes	Yes	Yes	Yes	ND	Yes	ND
O-glycosylation	Yes	Yes	Yes	ND	ND	ND	Yes
N-glycosylation	No	Yes	No	No	No	No	No
Phosphorylation	Yes	Yes	Yes	ND	ND	Yes	Yes

TABLE 1. **Properties of granins** (Taupenot *et al.*, 2003). ND = not determined.

Interestingly, granins not only serve as granule matrix. Indeed various biological activities have been attributed to peptides derived from granins. With the exception of SgIV (not determined), the granins have several dibasic sites that are potential cleavage sites for proteolytic processing. Full-length granins undergo partial proteolytic processing during their storage in secretory granules to yield a large array of biologically active peptides. Many of these have antifungal or antibacterial activity while some inhibit vasoconstriction or

hormone release (Taupenot *et al.*, 2003). Defined proteolytic products include chromofungin, vasostatin I+II, chromacin I+II, pancreastatin, and catestatin derived from CgA or chromacin and secretolytin derived from CgB. SgII is processed to secretoneurin which stimulates the release of dopamine and has chemoattractant effects on neighboring cells.

### 3.4.3 Processing Enzymes

Processing enzymes are considered as accessory proteins of secretory granules. Proprotein convertases (PCs) are required for the activation of a large array of precursor proteins. The substrates include precursors for neuropeptides, peptide hormones, growth and differentiation factors, adhesion proteins, membrane receptors, blood coagulation factors, plasma proteins, transcription factors, viral coat proteins, and bacterial toxins (Seidah and Chretien, 1999). Their discovery was based on findings in yeast genetics. The Kex2 peptidase, a serine-protease, was shown to be responsible for proteolytic processing of the alpha-mating factor precursor with specificity for cleaving on the carboxyl side of paired basic residues (Julius *et al.*, 1984). Sequence homology analysis revealed a mammalian homolog encoded by the fur gene which led to the identification of seven kexin-related mammalian enzymes, the family of proprotein convertases (Fig. 7). The members include furin (SPC1), PC2 (SPC2), PC3 (PC1 or SPC3), PACE4 (SPC4), PC4 (SPC5), PC6 (PC5 or SPC6), and PC7 (LPC, PC8 or SPC7) (Bergeron *et al.*, 2000).

PCs are highly selective endoproteases which cleave their substrates at specific dibasic sites. The consensus motif for processing is Arg-X-Lys/Arg-Arg↓. The first residue on the N-terminal side of the cleaved polypeptide is named P1, the second residue is P2, and so on. The amino acids on the C-terminal side are named P1', P2', P3', etc. The P4 Arg is not mandatory for cleavage, however, the presence of an Arg residue at position P6, P4 or P2 generally enhances cleavage (Hosaka *et al.*, 1991).



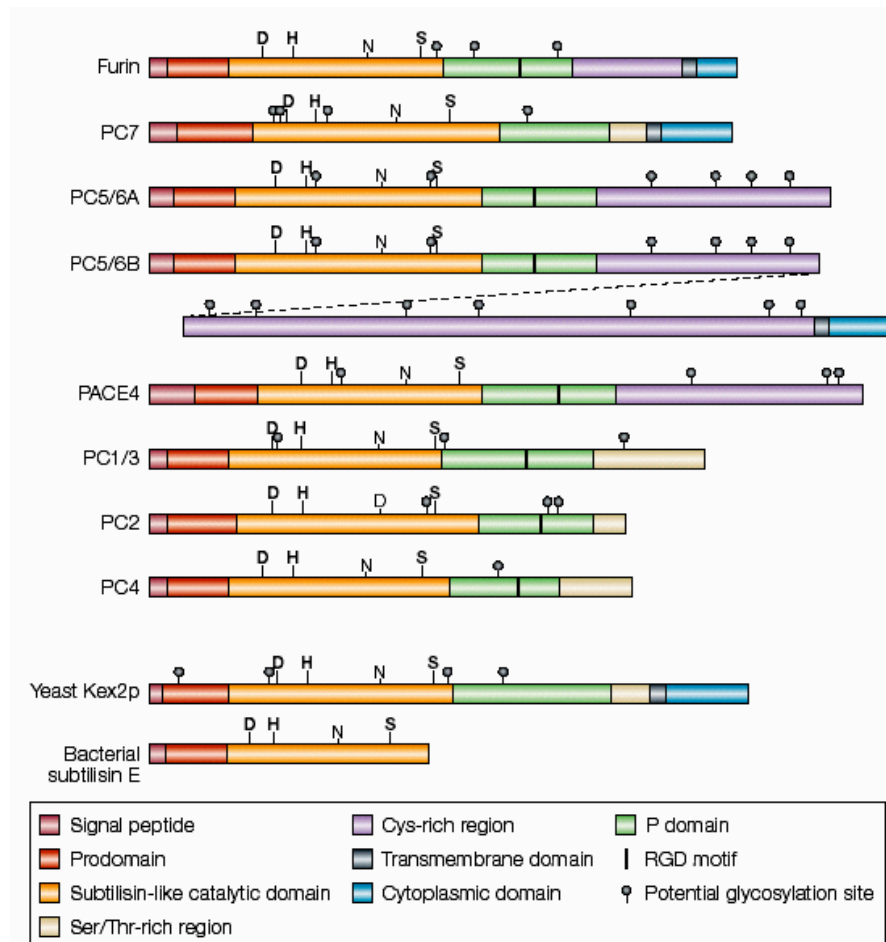


FIG. 7. **Structural comparison of the proprotein convertase family and their related yeast kexin and bacterial subtilisin proteases** (Thomas, 2002).

After endoproteolytic cleavage the free C-terminal basic residues are removed by members of the metallopeptidase family such as carboxypeptidase E (CPE) (Kemmler *et al.*, 1973) which is targeted to the regulated secretory pathway and acts in an endocrine-specific manner. Other members of the family are CPD (Song and Fricker, 1995) which plays a role in processing of proteins in the constitutive secretory pathway, CPZ (Song and Fricker, 1997) and CPM (McGwire and Skidgel, 1995).

#### Domain organization

All members of the PC family have a similar domain structure. An N-terminal signal peptide is followed by the pro-segment, the catalytic domain, the P domain and a more variable C-terminal domain which can include sorting signals, transmembrane and cytosolic domains, cysteine-rich regions, or amphipathic helices. PCs are initially synthesized as inactive pre-pro-proteins targeted to the ER by the signal peptide which is cleaved by a signal peptidase after the translocation across the ER membrane. In the next processing step the pro-segment is autocatalytically cleaved at a dibasic site in the ER. This cleavage is important for exiting the ER. PC2 is an exception in that it requires acidic conditions of the TGN and secretory granules for autoactivation (Lamango *et al.*, 1999). PC2 is also unique for its

interaction with the neuroendocrine protein 7B2 (SgV) in the ER which is important for transport and activity (Muller *et al.*, 1997). The pro-segments are suggested to act as intramolecular chaperones and are essential for proper folding of convertases. After cleavage the pro-segment stays attached to the convertase and inhibits PC activity via an interaction with the catalytic domain. In a later compartment of the secretory pathway proteolytic cleavage at another dibasic site of the pro-segment mediates its dissociation which results in PC activation. This second cleavage ensures that convertases are activated only after reaching their target compartment where the pH and  $\text{Ca}^{2+}$  concentrations are optimal for activity.

The catalytic domain contains the conserved catalytic triad residues Asp, His and Ser. The conformation of the catalytic domain is primarily responsible for substrate selectivity. The crystal structure of mouse furin and yeast kexin revealed the exact domain fold and the detailed architecture of the substrate binding cleft explaining the stringent requirement for basic residues by charge-complementary pockets. The active site cleft resembles a canyon-like crevice with a peptide loop on each side and the active site triad arranged in its center (Henrich *et al.*, 2003). Modeling of the structure of the other PCs revealed similar domain folds. Differences in substrate recognition can be explained by slight charge variations in the vicinity of the active site cleft (Henrich *et al.*, 2005). Mutational analysis of PCs confirmed that negatively charged residues of the catalytic pocket interact with positively charged residues of the substrate (Creemers *et al.*, 1993).

The P domain (or Homo B domain) is required for correct folding and stability of the convertase. The barrel-like P domain is folded separately and is covalently linked to the C-terminus of the spherical catalytic domain by an inter-domain linker. The two domains interact via hydrophobic interactions and a conserved salt bridge which is thought to be essential for the structural cohesion of PCs. All PCs, except PC7, contain a RGD motif which is usually involved in extracellular adhesion via integrins. The structural data, however, revealed that the Arg and Asp side-chains are exposed to different surfaces which makes integrin binding unlikely. Mutations on the RGD motif in PC3 had effects on substrate processing, precursor processing, enzyme stability and intracellular routing which could be explained by a structural destabilization due to modifications in the P-domain (Lusson *et al.*, 1997).

The C-terminal domain is quite variable among the family members. PC2, PC3 and PC4 contain a Ser/Thr-rich region and an amphipathic helix thought to be important for hydrophobic interactions with TGN and secretory granule membranes. Furin, PACE4, and PC6 have a Cys-rich domain which in PACE4 and PC6A was shown to be necessary for binding to the extracellular matrix and for substrate accessibility (Nour *et al.*, 2005). Furin, PC6B and PC7 have a transmembrane segment followed by a cytoplasmic tail. This domain affects cellular sorting and has been well studied for furin (Molloy *et al.*, 1999).

#### *Tissue distribution and subcellular expression*

Most cells do not express a single PC but rather a distinct mix thereof. Temporal and spatial specificity of expression patterns allows for availability in various proportions and combina-

tions at different locations. Overlapping cellular expression patterns raise the possibility of functional redundancy. Compensation of processing events has indeed been observed in PC2-null mice, where cleavage of proinsulin was partially compensated by other convertases (Furuta *et al.*, 1998). Similarly, the precursor of  $\beta$ -secretase (pro-BACE) is mainly processed by furin, but *in vivo* experiments showed that other PCs like PACE4, PC6A, PC6B and PC7 also process pro-BACE, although to a lesser extent (Creemers *et al.*, 2001). Despite functional redundancy overlapping functions of PCs seem to be minimized *in vivo* by the fact that they are either not coexpressed or that they are sorted to distinct cellular compartments. Based on their tissue distribution and intracellular localization PCs can be divided into four classes:

Class I: Furin and PC7 are ubiquitously expressed in mammalian cells. They both have a cytoplasmic tail containing sorting signals for routing between TGN, endosomes and the cell surface. Their strategic localization in the TGN and widespread expression patterns allow for cleavage of various substrates that travel to the cell surface via the constitutive secretory pathway.

Class II: PC2, PC3 and PC6A process regulated secretory proteins. They are primarily expressed in endocrine and neuronal cells. Since they process prohormones and proneuropeptides in the TGN or immature secretory granules they are sometimes referred to as prohormone convertases. PC2 and PC3 have a differential expression pattern with a varying relative ratio which can result in different biologically active compounds derived from a common proneuropeptide.

Class III: PACE4 and PC6 are ubiquitously expressed and play a role in both the regulated and the constitutive secretory pathway. PACE4 expression was observed in the TGN and at the cell surface. PC6 exists as two C-terminal variant isoforms, named PC6A and PC6B, as a result of differential mRNA splicing. PC6A is a soluble luminal protein targeted to the regulated secretory pathway, while PC6B has a transmembrane domain followed by a cytoplasmic tail and is mainly located in a Golgi compartment communicating with endosomes (Xiang *et al.*, 2000). A processed version of PC6B can be secreted, and both PC6A and PC6B are processed at their C-termini to produce an even shorter, secreted form lacking the cell surface anchored cystein-rich domain (De Bie *et al.*, 1996). PACE4 expression is very high in some restricted areas of the central nervous system and low in other regions. PACE4 and PC6 generally have non-overlapping expression patterns and are thought to be involved in the processing of a distinct set of proneuropeptides in the nervous system and proproteins in peripheral tissues during development (Zheng *et al.*, 1997). PACE4 plays an important role in patterning the early mouse embryo via TGF $\beta$ -related signaling events (Constam and Robertson, 2000).

Class IV: PC4 has a unique expression pattern and is restricted to testicular germ cells. Subcellular expression was observed in acrosomal granules of spermatides and on the sperm plasma membrane (Gyamera-Acheampong *et al.*, 2005). It is thought to play a role in the production of fertile and developmentally competent spermatozoa.

### 3.4.4 Other Granule-associated Proteins

While hormones, neuropeptides, granins and prohormone convertases are efficiently sorted into the regulated secretory pathway other proteins may be dragged into secretory granules only to a limited extent and fulfill additional functions in other subcellular compartments. Various transporters are found in the membranes of secretory granules including the H<sup>+</sup>-translocating ATPase for acidification of the matrix, the H<sup>+</sup>/chatecholamine antiporter, the vesicular monoamine transporter (VMAT), and the IP3-Receptor (IP3-R)/Ca<sup>2+</sup> channel (Yoo, 1994; Apps, 1997).

The function of the IP3-R is not exclusive to the regulated secretory pathway. Intracellular Ca<sup>2+</sup> is mobilized by the signaling molecule inositol 1,4,5-trisphosphate (IP3) which interacts with the IP3-R to release Ca<sup>2+</sup> from intracellular stores. IP3-R is mainly located in ER membranes where it mediates Ca<sup>2+</sup> mobilization from the ER lumen into the cytoplasm in response to various external stimuli. It exists as three isoforms, derived from three distinct genes. Type 1 IP3-R is mainly expressed in brain tissue and plays a role in motor and learning systems (Nishiyama *et al.*, 2000). Type 2 and 3 IP3-Rs are expressed in various tissues and mediate exocrine secretion of digestive enzymes (Futatsugi *et al.*, 2005). Secretory granules contain ~40 mM Ca<sup>2+</sup> (the ER ~3 mM) and store up to 60% of intracellular calcium. Intragranular Ca<sup>2+</sup> is largely bound to chromogranins which is why granins were suggested to act as Ca<sup>2+</sup> storage proteins. CgA and CgB were shown to directly interact with the IP3-R at an intragranular pH of 5.5 and to activate the IP3-R/Ca<sup>2+</sup> channel (Yoo *et al.*, 2002). All three subtypes of the IP3-R were shown to be targeted to newly formed secretory granules induced by CgA and CgB in NIH3T3 (Huh *et al.*, 2005) fibroblast cells with IP3-R levels similar to the those found in secretory granules of neuroendocrine chromaffin cells. Secretory granules might act as an important calcium storage organelle, besides the ER, in cells specialized for secretion. Moreover, IP3-R has been found in the nucleoplasm where it was proposed to act in a complex with CgB and phospholipids to regulate Ca<sup>2+</sup> release from a putative nucleoplasmic store (Huh *et al.*, 2005).

Cytochrome b561 and some other enzymes isolated from secretory granules are associated with redox functions (Apps, 1997). However, the role of several other membrane proteins of secretory granules (synaptophysin, glycoproteins, ATPase II) has remained unclear.

## AIM OF THIS THESIS

We have observed that prohormones and granins are capable of inducing the formation of granule-like structures in constitutively secreting cells. The characterization of these structures revealed that they represent discrete organelles. The data suggest that the formation of secretory granules is a self-assembly process which does not rely on an endocrine-specific machinery. Alternatively, the required machinery could be latently expressed in all cells.

The goal of this work was to define the similarities and differences between granule-like structures – induced by prohormones, granins, and prohormone convertases (PCs) in non-endocrine cells – and real secretory granules of endocrine cells. Our studies address the morphology of granule-like structures and their competence for storage and regulated release of cargo proteins. We have analyzed various regulated secretory proteins for their potency to induce granule-like structures, and we observed considerable differences in efficiency of induction and in morphology of the structures. The granulogenic effects vary considerably depending on the type of protein expressed.

PCs are particular as they were shown to interact with membranes. This feature has been postulated to mediate targeting to the regulated secretory pathway (Creemers *et al.*, 1996; De Bie *et al.*, 1996; Jutras *et al.*, 2000). PC3 has even been claimed to be a transmembrane protein exposing its C-terminal tail to the cytosol (Arnaoutova *et al.*, 2003b). We were intrigued by this suggestion, considering that the proposed transmembrane region contained a high number of charged amino acids while typical transmembrane segments consist of uncharged, mainly hydrophobic residues. Since the mode of membrane interaction has significant implications for the mechanism of sorting we analyzed the membrane integration of PC3. Membrane integrity and the topology of PC3 were assessed by various biochemical assays.



## CHAPTER I

### **Expression of Regulated Secretory Proteins Is Sufficient to Generate Granule-like Structures in Constitutively Secreting Cells**

The Journal of Biological Chemistry, 2004, Volume 279, 20242-20249

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<sup>1</sup> Work contributed to this publication is depicted in figures 10 and 12.

## Abstract

The formation of secretory granules and regulated secretion are generally assumed to occur only in specialized endocrine, neuronal, or exocrine cells. We discovered that regulated secretory proteins such as the hormone precursors pro-vasopressin, pro-oxytocin, and pro-opiomelanocortin, as well as the granins secretogranin II and chromogranin B, but not the constitutive secretory protein  $\alpha_1$ -protease inhibitor, accumulate in granular structures at the Golgi and in the cell periphery in transfected COS-1 fibroblast cells. The accumulations were observed in 30–70% of the transfected cells expressing the prohormones and for virtually all cells expressing the granins. Similar structures were also generated in other cell lines believed to be lacking a regulated secretory pathway. The accumulations resembled secretory granules morphologically in immunofluorescence and electron microscopy. They were devoid of markers of the endoplasmic reticulum, endosomes, and lysosomes, but in part stained positive for the trans-Golgi network marker TGN46, consistent with their formation at the trans-Golgi network. When different regulated proteins were coexpressed, they were frequently found in the same granules, whereas  $\alpha_1$ -protease inhibitor could not be detected in accumulations formed by secretogranin II, demonstrating segregation of regulated from constitutive secretory proteins. In pulse-chase experiments, significant intracellular storage of secretogranin II and chromogranin B was observed, and secretion of retained secretogranin II was stimulated with the calcium ionophore A23187. The results suggest that expression of regulated cargo proteins is sufficient to generate structures which resemble secretory granules in the background of constitutively secreting cells, supporting earlier proposals on the mechanism of granule formation.



## Introduction

Endocrine and neuroendocrine cells possess a regulated secretory pathway in addition to the constitutive pathway present in all cells (Burgess and Kelly, 1987). At the trans-Golgi network (TGN), regulated cargo protein, such as peptide hormone precursors and granins, are sorted into secretory granules where they are stored in a densely packed form. By an external stimulus, the granules are triggered to fuse with the plasma membrane and to release their contents in a controlled manner. The regulated secretory pathway thus requires mechanisms to segregate regulated cargo from constitutively secreted proteins and to package them into specialized vesicles. These membrane-bounded organelles in addition recruit prohormone processing enzymes as well as the components necessary for luminal acidification, for transport of the granules to the cell periphery or along the axon, and for the controlled fusion with the plasma membrane.

So far, little is known about the machinery that is required to generate secretory granules. Two non-exclusive models have been proposed on how secretory granules are formed and how specific cargo selection is accomplished (Arvan and Castle, 1998; Tooze, 1998). The first model, termed *sorting-for-entry*, is analogous to receptor-mediated endocytosis and mannose-6-phosphate receptor-dependent lysosomal transport (Schmid, 1997) where cargo binds to receptors which in turn recruit a cytosolic coat. Similarly, regulated secretory proteins may be selected and other proteins excluded by interaction with receptors in the TGN membrane prior to granule formation. Consistent with this model, the propeptide of prosomatostatin (Stoller and Shields, 1989) and a disulfide-bonded loop segment of CgB (Kromer *et al.*, 1998; Glombik *et al.*, 1999) have been shown to be necessary and sufficient to mediate granule sorting, suggesting they constitute sorting signals. An amphipathic loop of pro-opiomelanocortin (POMC) was also found to be necessary for sorting (Cool *et al.*, 1995). Carboxypeptidase E was reported to bind to this loop and to be required for granule sorting (Cool *et al.*, 1997). Its proposed role as a sorting receptor, however, is controversial (Irminger *et al.*, 1997). The apparent substoichiometric amount of putative sorting receptors in secretory granules may be explained by the tendency of regulated secretory proteins to aggregate under the conditions of the trans-Golgi (low pH and high calcium concentrations) (e.g. ref. Chanat and Huttner, 1991; Colomer *et al.*, 1996)), which would allow each receptor to sort an entire polymer of cargo molecules.

The alternative model, *sorting-by-retention*, proposes that selective aggregation of regulated cargo in the TGN results in an immature granule. Captured non-granule molecules are subsequently removed in vesicles budding from maturing secretory granules by clathrin-coated vesicles and by so-called constitutive-like secretion, whereas specific granule cargo is retained (Kuliawat and Arvan, 1992). This explains the presence of the mannose 6-phosphate receptor, clathrin and AP-1 adaptors on immature secretory granules (Dittie *et al.*, 1996; Klumperman *et al.*, 1998).

Recently, it has been proposed that a single protein of endocrine and neuronal cells, chromogranin A (CgA), controls secretory granule biogenesis (Kim *et al.*, 2001). Expression of CgA was even found to induce granular structures in transfected CV-1 fibroblast cells. The interpretation of these observations has been discussed controversially (Day and Gorr, 2003;

Huh *et al.*, 2003; Kim *et al.*, 2003). In the alternative view, condensing CgA might act as an aggregation or assembly factor similarly to the *sorting-by-retention* model (Day and Gorr, 2003).

In this study, we report that several other cargo proteins of the regulated secretory pathway of endocrine cells, peptide hormone precursors as well as granins, induce the formation of granule-like structures when expressed in cell lines normally lacking regulated secretion. Expression of cargo is sufficient to drive segregation of regulated and constitutive secretory proteins and the formation of dense, membrane-bounded accumulations with similar ultrastructural appearance as secretory granules, suggesting that initial granule formation requires no additional machinery specific to regulated secretory cells besides the regulated cargo itself.

## Materials and Methods

*cDNA constructs* — cDNAs for regulated secretory proteins were gifts by G. Boileau (University of Montreal; porcine POMC), H. Gerdes (University of Heidelberg; human SgII and CgB), and M. Ito (Northwestern University, Chicago; human vasopressin). The coding sequence of human oxytocin was assembled from the exons amplified by polymerase chain reaction from the gene (a gift by J. Amico, University of Pittsburgh). The cDNA of human  $\alpha_1$ -protease inhibitor (A1Pi) was from J.L. Brown (University of Colorado, Denver). To C-terminally tag proteins with a c-myc epitope, a Kpn I restriction site was introduced by polymerase chain reaction in place of the stop codon for ligation to the myc epitope sequence encoding EQKLISEEDLN<sub>stop</sub>. In the same way, the C1 epitope ETELDKASQEPPLL<sub>stop</sub> corresponding to the C-terminal sequence of the human asialoglycoprotein receptor H1, for which we have a rabbit anti-peptide antiserum (Geffen *et al.*, 1993), was fused to CgB and SgII. The cDNAs were subcloned into the SV40-based expression plasmids pECE or pCB6. For expression in COS-1 cells without plasmid amplification, SgII-myc was also cloned into pcDNA5 (Invitrogen), which lacks SV40 sequences.

*Cell culture and transfection* — Madin-Darby canine kidney (MDCK) strain II and HepG2 hepatoma cells were grown in minimal essential medium, COS-1, chinese hamster ovary CHO-K1, human embryo kidney HEK293, and NIH/3T3 cells in Dulbecco's minimal essential medium, supplemented with 2mM L-glutamine, 100 units/ml penicillin, 100 $\mu$ g/ml streptomycin, and 10% fetal calf serum at 37°C with 7.5% CO<sub>2</sub>. Cells were transfected using Lipofectin (Life Technologies) with 2  $\mu$ g plasmid DNA/35-mm dish at ~40% confluency and processed after 48 h. To reduce expression levels of SgII-myc in COS cells, the SgII-myc expression plasmid was gradually diluted with pEGFP-N1 (Clontech), whereby the total amount of plasmid was kept constant at 2  $\mu$ g/35-mm plate. Expression levels were assayed by immunoblot analysis using rabbit anti-secretoneurin antiserum (Kirchmair *et al.*, 1993), a gift from R. Fischer-Colbrie (University of Innsbruck), at a dilution of 1:1000. To generate stable cell lines, HEK293 cells were transfected with pCB6 containing the vasopressin precursor cDNA und subjected to selection with 1 mg/ml G418-sulfate (Gibco). Resistant lines were cloned and analyzed by immunoblotting and immunofluorescence microscopy.

*Immunofluorescence and antibodies* — We used rabbit anti-neurophysin II antibodies against pro-vasopressin (which also recognizes neurophysin I of pro-oxytocin) from ICN, rabbit anti-human adrenocorticotrophic hormone (ACTH) to detect POMC from Sigma, sheep anti-human TGN46 from Serotech, rabbit anti-A1Pi antiserum from Jerry L. Brown (UCHSC, Denver), rabbit anti-human EEA1 (early endosome antigen 1) antiserum from H. Stenmark (Norwegian Radium Hospital, Oslo), rabbit anti-protein disulfide isomerase from H.P. Hauri (Biozentrum, Basel), monoclonal antibodies against the c-myc epitope (9E10) (Evan *et al.*, 1985), against giantin from H.P. Hauri, against lamp-1 from J. Rohrer (FMI, Basel), against rab5 from R. Jahn (MPI, Göttingen), and a rabbit anti-peptide antiserum recognizing the C1-epitope (Geffen *et al.*, 1993). Antibodies were used at a dilution of 1:100 except for anti-ACTH

(1:200) and anti-rab5 (1:80). As secondary antibodies, non-crossreacting cy3-labeled goat anti-mouse, cy2-labeled goat anti-rabbit, cy3-labeled donkey anti-sheep, and cy2-labeled donkey anti-rabbit immunoglobulin antibodies (from Jackson ImmunoResearch and Amersham Biosciences) were used as appropriate according to the manufacturers' recommendations.

Cells were grown on 14-mm glass coverslips, fixed with 3% paraformaldehyde for 15 min at room temperature, washed in phosphate-buffered saline (PBS), quenched with 50 mM  $\text{NH}_4\text{Cl}$  in PBS, and permeabilized with 0.1% Triton X-100 for 10 min. Nonspecific antibody binding was blocked with PBS containing 1% bovine serum albumin. The fixed cells were incubated at room temperature with primary antibodies for 1 h, washed with PBS with albumin, and stained with fluorescent secondary antibodies in PBS with albumin for 30 min. After several washes with PBS with albumin, PBS, and water, the cover slips were mounted in Mowiol 4-88 (Hoechst). Staining patterns were analyzed using a Zeiss Axioplan 2 microscope with a KX Series Imaging System (Apogee Instruments) or a Zeiss Axiovert 200M confocal LSM 510 Meta microscope.

*Electron microscopy* — To enrich transfected cells for ultrastructural analysis, COS-1 cells were co-transfected with an expression plasmid encoding pro-vasopressin or SgII-myc, and a plasmid pEGFP-N1 encoding green fluorescent protein (GFP). The cells were brought into suspension by trypsinization and were sorted for GFP fluorescence using a MoFlo cell sorter (Cytomation, Fort Collins, CO). They were then fixed with 3% paraformaldehyde/0.5% glutaraldehyde in PBS, pH 7.4, for 1 h at room temperature, washed with PBS, incubated with 1% osmium tetroxide for 1 h, washed with water, and dehydrated by successive 15-min incubations with 50%, 70%, 90%, 100% ethanol, followed by 1 h with Epon/acetone 1:1, 1 h with Epon/acetone 2:1, and two times 2 h with Epon, and 24–48 h at 60°C. Thin sections were stained with 6% uranyl acetate for 1 h and with lead acetate for 2 min.

For immunogold labeling, GFP-positive cells expressing SgII-myc were fixed with 3% paraformaldehyde/0.5% glutaraldehyde in PBS for 1 h, washed with PBS, fixed again with 0.5% osmium tetroxide for 1 h, washed with water, and incubated 15 min each with 50% and 70% ethanol, 1 h with ethanol/LR White 2:1, 1 h with LR White (from Polysciences, Warrington, PA), and 24–48 h at 60°C. Thin sections were then blocked twice for 5 min with 2% BSA in PBS, incubated with anti-myc antibody 9E10 in 2% BSA for 3 h, washed three times for 10 min with PBS and again two times for 5 min with 2% BSA, incubated for 1 h with goat anti-mouse immunoglobulin (from British Biocell) coupled to 10-nm gold, washed with PBS and with water and contrasted with 6% uranyl acetate for 1 h and with lead acetate for 2 min.

*Storage and stimulation assays* — To analyze the secretion behavior, COS-1 cells transfected with A1Pi<sup>TS</sup> (Leitinger *et al.*, 1994), CgB-C1, or SgII-C1 were labeled with [<sup>35</sup>S]sulfate (0.5 mCi/ml, from Amersham, in sulfate-free medium, from Gibco) for 90 min at 19°C, followed by a chase at 37°C for up to 6 h in medium containing excess unlabeled sulfate. The medium was removed and replaced by fresh chase medium at different times. The labeled protein secreted into the medium as well as that retained in the cells at the end of the experiment

was immunoprecipitated and analyzed by gel electrophoresis and autoradiography. Signals were quantified by phosphorimager.

To analyze stimulated secretion, COS-1 cells expressing SgII-C1 were labeled with [<sup>35</sup>S]sulfate for 90 min at 19°C, followed by a chase of 3 h, after which the medium was replaced by medium with or without 1  $\mu$ M A23187 (Sigma; prepared by adding a 100-fold concentrated stock solution in DMSO or just DMSO to the medium). Labeled protein secreted into the medium during this time was analyzed by immunoprecipitation, gel electrophoresis, and autoradiography. The total amount of labeled SgII-C1 was determined by immunoprecipitation from the medium and the cell lysate of parallel aliquots of transfected cells after the labeling period. Cell integrity as judged by trypanblue exclusion was not affected after 30 min with 1  $\mu$ M A23187.

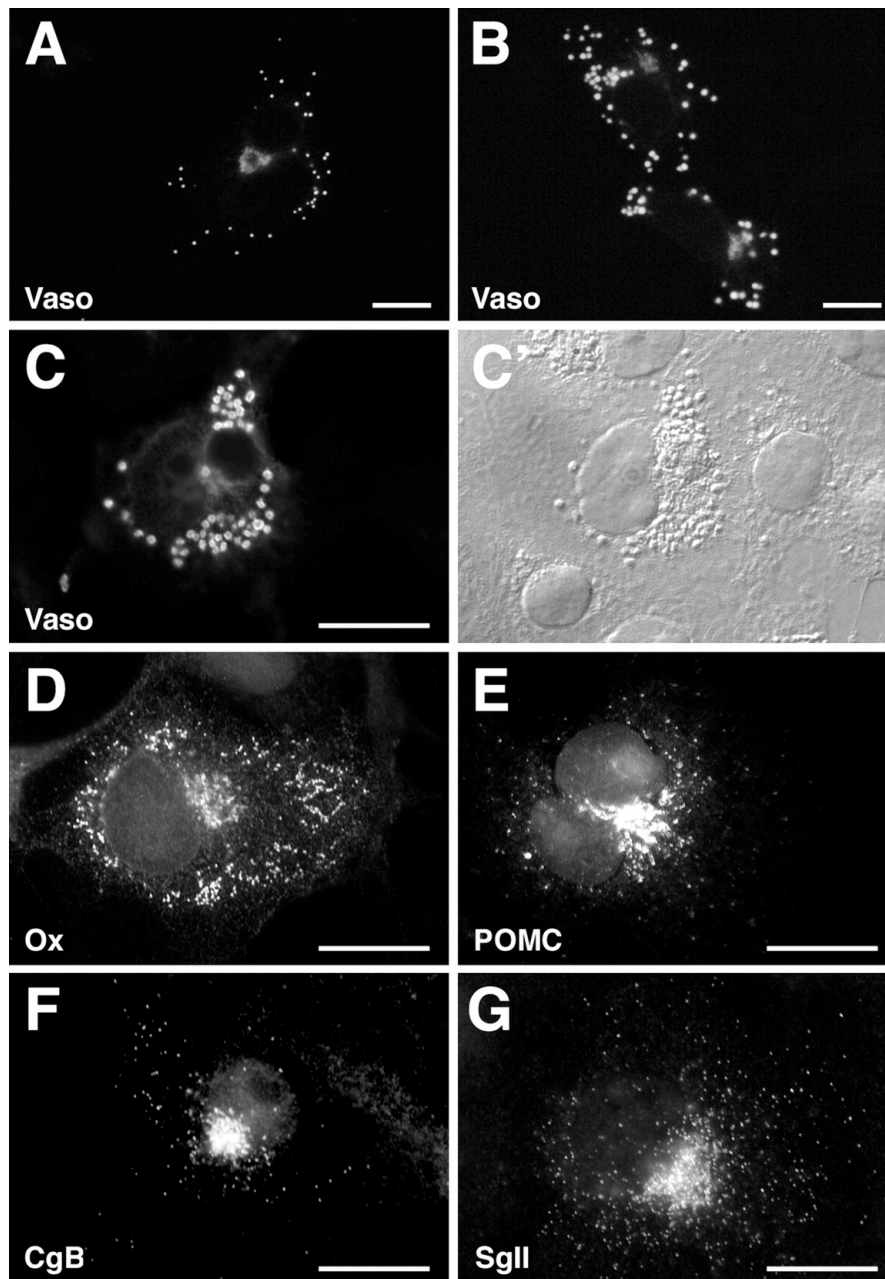
To estimate the effect of stimulation on the density of granule-like structures, transfected COS-1 cells expressing SgII-myc were incubated for 30 min with fresh medium with or without 1  $\mu$ M A23187, and then fixed and processed for immunofluorescence. Fifty random cells of each condition were photographed and the granule-like structures counted, excluding the Golgi/TGN area of the cells where structures could not be separated from each other, by a person unaware of the sample identity. The numbers were normalized for the size of the cells as estimated by measuring the area from tracings of the cell outlines excluding the Golgi area using Adobe Photoshop.

## Results

*Pro-vasopressin expressed in COS-1 cells accumulates in granular structures* — The vasopressin precursor protein is normally expressed in specific neurons of the hypothalamus. Upon translocation into the endoplasmic reticulum (ER), it is transported through the secretory pathway to the TGN where it is packaged into immature secretory granules. The precursor is processed by prohormone convertases to the nonapeptide hormone, the carrier protein neurophysin II, and a C-terminal glycopeptide. Granules are transported to the nerve terminals in the neurohypophysis and its contents are released as the granule membrane is triggered to fuse with the plasma membrane.

Upon expression in transfected COS-1 cells, which lack a regulated secretory pathway, pro-vasopressin is not proteolytically cleaved and is in its majority constitutively secreted (~85% in 2h) (Beuret *et al.*, 1999). When subjected to indirect immunofluorescence analysis using an antibody directed against neurophysin II, many transfected cells showed the expected weak staining of the ER typical of a secretory protein whose rate-limiting step in secretion is protein folding and ER exit. In approximately 50% of the expressing cells, however, intense additional staining was found in punctate patterns, often near the nucleus, but frequently also in the periphery of the cells (Fig. 8A–C). The fluorescent structures varied in apparent size between less than 0.5  $\mu\text{m}$  and up to 4  $\mu\text{m}$  in diameter.

In bright-field microscopy using differential interference contrast, the larger vasopressin accumulations were visible as prominent structures (Fig. 8C'), indicating that they are particularly dense. Cells expressing pro-vasopressin in such accumulations can often be identified and distinguished from nontransfected cells by bright-field microscopy alone (Fig. 8C', compare the transfected cells with the surrounding untransfected ones). This indicates that the dense structures are newly generated by pro-vasopressin expression and do not constitute pre-existing compartments which receive pro-vasopressin when expressed. The appearance and density of these elements are reminiscent of secretory granules of endocrine cells and raise the possibility that expression of this regulated secretory protein induces the formation of similar, granule-like structures even in a cell line with only a constitutive secretory pathway.



**FIG. 8. Regulated secretory proteins expressed in transfected COS-1 cells accumulate in granular accumulations.**

Transfected COS-1 cells expressing pro-vasopressin (A–C), pro-oxytocin (D), POMC (E), CgB-myc (F), and SgII-myc (G) were analyzed by indirect immunofluorescence using antibodies against neurophysin, ACTH, and the myc epitope, respectively. In panel C', the same field as in C is shown by differential interference contrast microscopy. The micrographs are representative of ~50%, ~30%, and ~75% of the cells expressing pro-vasopressin, pro-oxytocin, or POMC, respectively. The other cells showed low staining of ER and Golgi, as expected for a constitutively secreted protein. Essentially all cells expressing CgB-myc or SgII-myc showed the granular accumulations as shown in panels F and G. Bars, 20  $\mu$ m.

*Granule-like structures are formed by various regulated cargo proteins* — To test whether the ability to form granule-like structures is unique to the vasopressin precursor, the cDNAs of other granule cargo were transiently expressed in COS-1 cells and analyzed by immunofluorescence. Pro-oxytocin is closely related to pro-vasopressin with 74% identity. In approximately one third of COS-1 cells expressing pro-oxytocin, a punctate pattern of accumulated protein was detected (Fig. 8D), very similar to the finer structures generated by pro-vasopressin. POMC, the precursor of ACTH and other active peptides of the anterior pituitary gland, produced small granular structures in ~75% of the transfected cells (Fig. 8E).

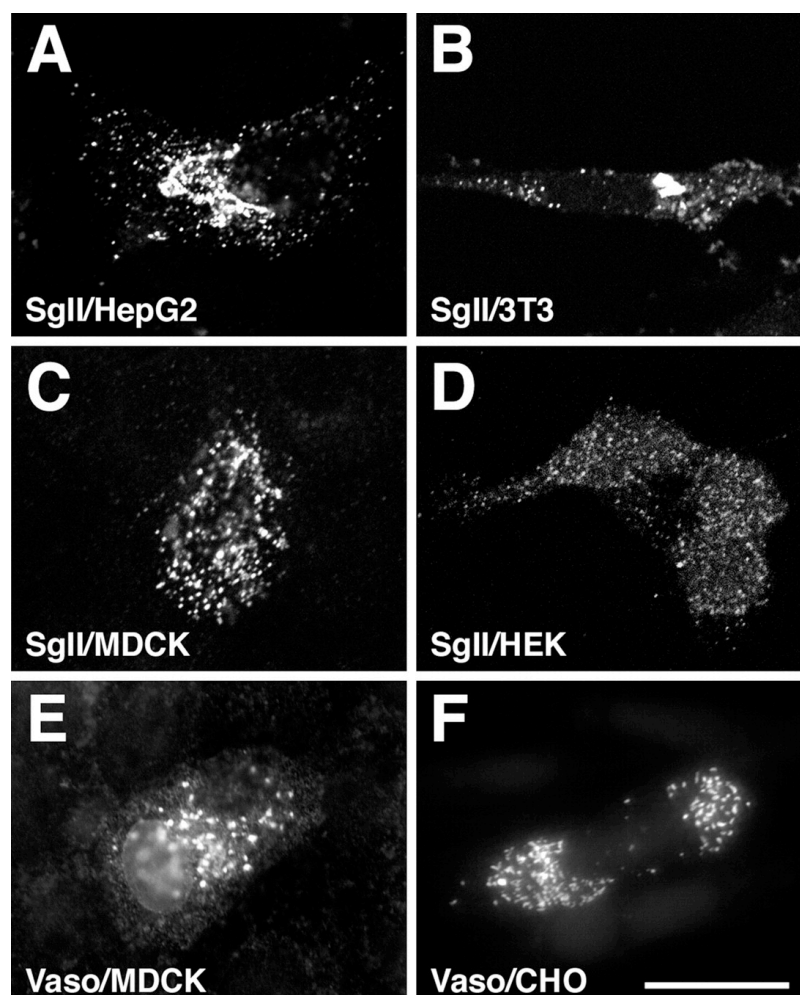
We further tested chromogranin B (CgB) and secretogranin II (SgII), two members of the granin family of acidic proteins found in a variety of endocrine and neuronal cells (Huttner *et al.*, 1991). These proteins are believed to function as helper proteins for the packaging of peptide hormones and neuropeptides (Rosa *et al.*, 1985). In transfected COS-1 cells, both CgB and SgII, tagged with a myc-epitope for detection, generated a multitude of small granular structures in virtually all expressing cells (Fig. 8F–G).

*Formation of granular structures is not specific to COS cells or to high overexpression* — The formation of these accumulations in COS-1 cells may be due to the latent expression of the putative machinery to generate secretory granules and/or to the high expression levels obtained by T antigen-mediated amplification of the SV40-based expression plasmid used. To test this, SgII-myc or pro-vasopressin were transfected into HepG2, NIH/3T3, MDCK II, HEK293, or CHO-K1 cells. In all cell lines, similar accumulations were observed as in COS-1 cells (Fig. 9).

To test the influence of the expression level, COS-1 cells were transfected with the SgII-myc plasmid increasingly diluted with an unrelated plasmid (Fig. 10A, lanes 1–4) or using an expression plasmid lacking SV40 origin of replication and promoter sequences (pcDSg; lane 5). As judged by immunoblot analysis, the expression level was reduced at least 20-fold relative to standard conditions. For comparison, an aliquot of PC12 cell lysate was analyzed in parallel (lane 6). Immunofluorescence microscopy showed that the transfection efficiency was unchanged under these conditions (~10%) and that virtually all cells expressing SgII-myc produced granule-like structures independently of the expression level (Fig. 10A). Correcting the immunoblot signals for transfection efficiency and taking into account efficient storage in PC12 cells, the result suggests that formation of granular structures in COS-1 cells does not require massive overexpression of SgII in comparison to natural secretory granule-producing cells.

Furthermore, we generated clonal HEK293 cell lines stably expressing different levels of vasopressin precursor (Fig. 10B). In all lines, 40–80% of the cells showed granular accumulations without correlation to expression levels. Interestingly, none of the lines showed the massive structures of more than 2  $\mu\text{m}$  often observed in transient transfections, suggesting that they might be toxic. While the formation of vasopressin accumulations does not depend strongly on expression level, it is unknown why it is triggered in only a fraction of the cells.





**FIG. 9. Granular structures are formed in different cell types.**

HepG2 (A), NIH/3T3 (B), MDCK(C and E), HEK293 (D), and CHO-K1 cells (F) were transfected with a plasmid encoding SgII-myc (A–D) or the vasopressin precursor (E and F) and stained with anti-myc or anti-neurophysin antibodies and a cy3- or cy2-labeled secondary antibody, respectively. The confocal microscope was used for panels A–D). Bar, 20  $\mu$ m.

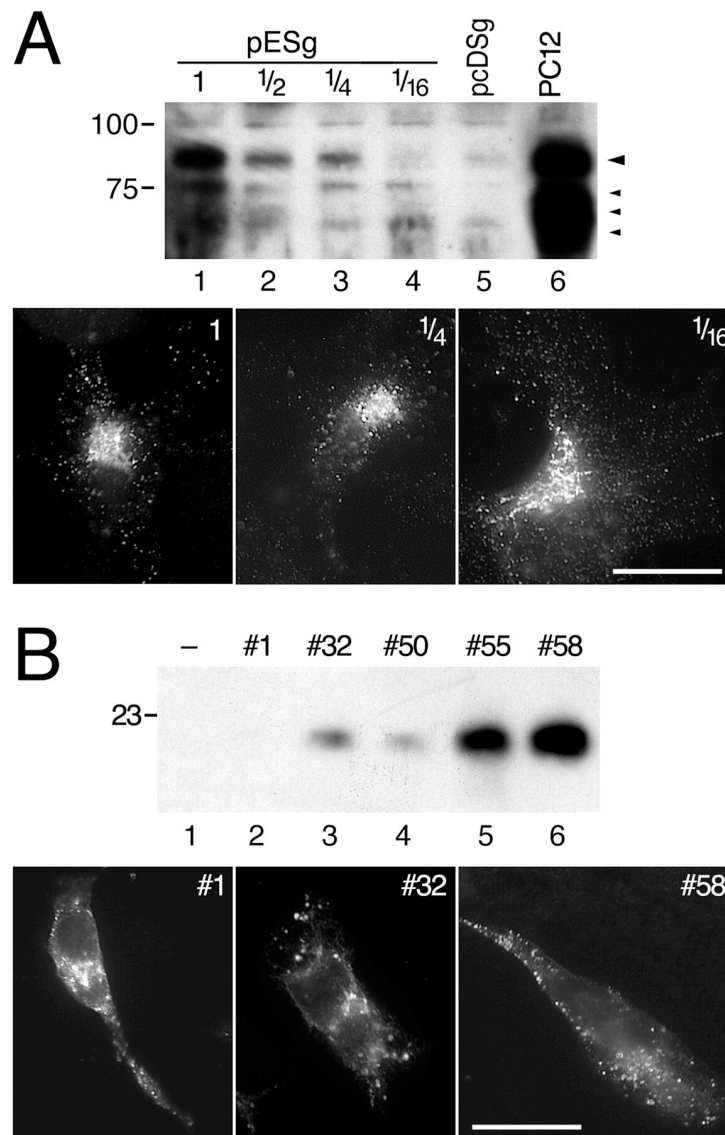
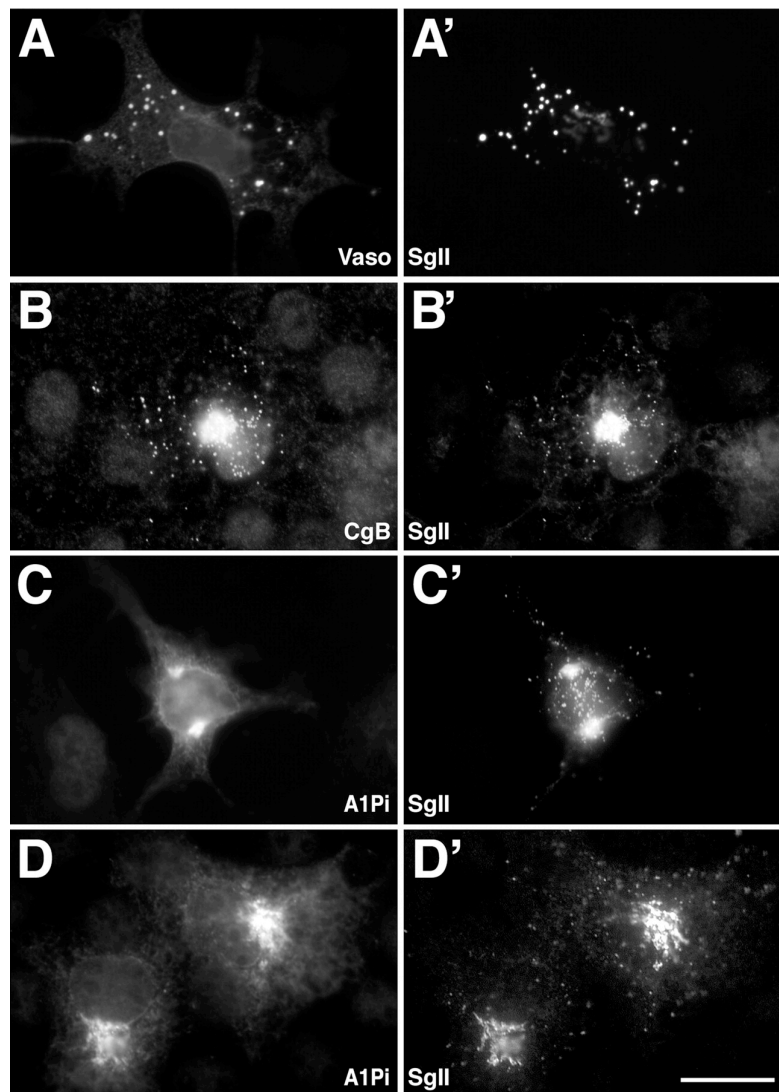


FIG. 10. Granule-like structures are not the result of excessive expression levels.

(A) COS-1 cells were transfected with pESg, the cDNA of SgII-myc in the SV40-based expression plasmid pECE, undiluted (lane 1), or with 2-, 4-, or 16-times less pESg diluted with pEGFP-N1 (a GFP encoding SV40-based plasmid; lanes 2–4). In addition, COS-1 cells were transfected with SgII-myc cDNA in pcDNA5 lacking SV40 sequences (pcDSg; lane 5). 30  $\mu$ g of cellular protein were analyzed by gel electrophoresis and immunoblotting. For comparison, 10  $\mu$ g of PC12 cell protein was analyzed in parallel (lane 6). The position of full-size SgII and its processing products are pointed out by large and small arrowheads, respectively. Molecular weight markers are indicated in kDa. In parallel, cells transfected with pESg alone (labeled 1) or with pESg diluted 4- ( $1/4$ ) or 16-fold ( $1/16$ ) with pEGFP-N1 were analyzed by immunofluorescence using anti-myc and cy3-labeled secondary antibodies. All cells expressing SgII-myc showed granule-like structures. (B) Stable HEK293 cell lines expressing vasopressin precursor were analyzed by Western analysis (30  $\mu$ g of cellular protein per lane; –, untransfected HEK293 cells) and by immunofluorescence. For all cell lines, 40–80% of the cells showed granular accumulations of pro-vasopressin without correlation to the expression levels. Bar, 20  $\mu$ m.

*A constitutive cargo protein is segregated from granule-like structures* — When pro-vasopressin was coexpressed with SgII-myc in COS-1 cells, the two proteins colocalized to the same structures (Fig. 11A and A'). Similarly, colocalization was observed for coexpressed CgB-myc and SgII-C1 (SgII tagged with the 10-amino acid epitope C1; Fig. 11B and B'). In contrast, when a constitutively secreted protein, A1Pi, was expressed in COS-1 cells, the cells showed reticular ER and perinuclear Golgi staining. Even when expressed in cells producing SgII-myc, A1Pi (Fig. 11C and D) was not detectable in the SgII-myc accumulations (C' and D'). These results suggest that the formation of granule-like structures is specific for regulated cargo and that there is segregation of constitutive and regulated secretory proteins.



**FIG. 11. Regulated proteins colocalize in granule-like structures whereas a constitutive cargo protein, A1Pi, is segregated.**

Transfected COS-1 cells expressing both pro-vasopressin and SgII-myc (A and A'), CgB-myc and SgII-C1 (B and B'), or A1Pi and SgII-myc (C, D and C', D'), were double-stained with antibodies against the two cargo proteins and appropriate cy2- or cy3-labeled secondary antibodies. Bar, 20  $\mu$ m.

*Granule-like structures are post-Golgi organelles* — In Fig. 12, the colocalization of organelle markers with SgII accumulations was analyzed to test whether the protein might form undegradable accumulations in the ER (e.g. due to misfolding), in lysosomes, or compartments en route. Antibodies against the ER chaperone protein disulfide isomerase did not colocalize with the punctate structures containing SgII (Fig. 12A). Outside the compact Golgi area, SgII structures were devoid of giantin (Fig. 12B), a marker of the Golgi stacks, and no colocalization was observed with the early endosome markers EEA1 (Fig. 12C) or rab5 (Fig. 12D), or with lamp-1 (lysosome-associated membrane protein-1; Fig. 12E), a marker for late endosomes and lysosomes.

Immunolocalization of the TGN marker TGN46 in COS-1 cells expressing SgII-myc showed the expected strong colocalization in the TGN and some staining of the granule-like structures near the center of the cell, but less or no TGN46 staining in peripheral SgII accumulations (Fig. 12F–F''). This is consistent with the model that these structures are derived from the TGN, but are losing TGN46 progressively by the budding of vesicles for constitutive-like secretion. The same conclusions were obtained from double-labeling experiments for pro-vasopressin structures and the ER marker p63, giantin, rab5, transferrin receptor, lamp-1, or TGN46 (data not shown).



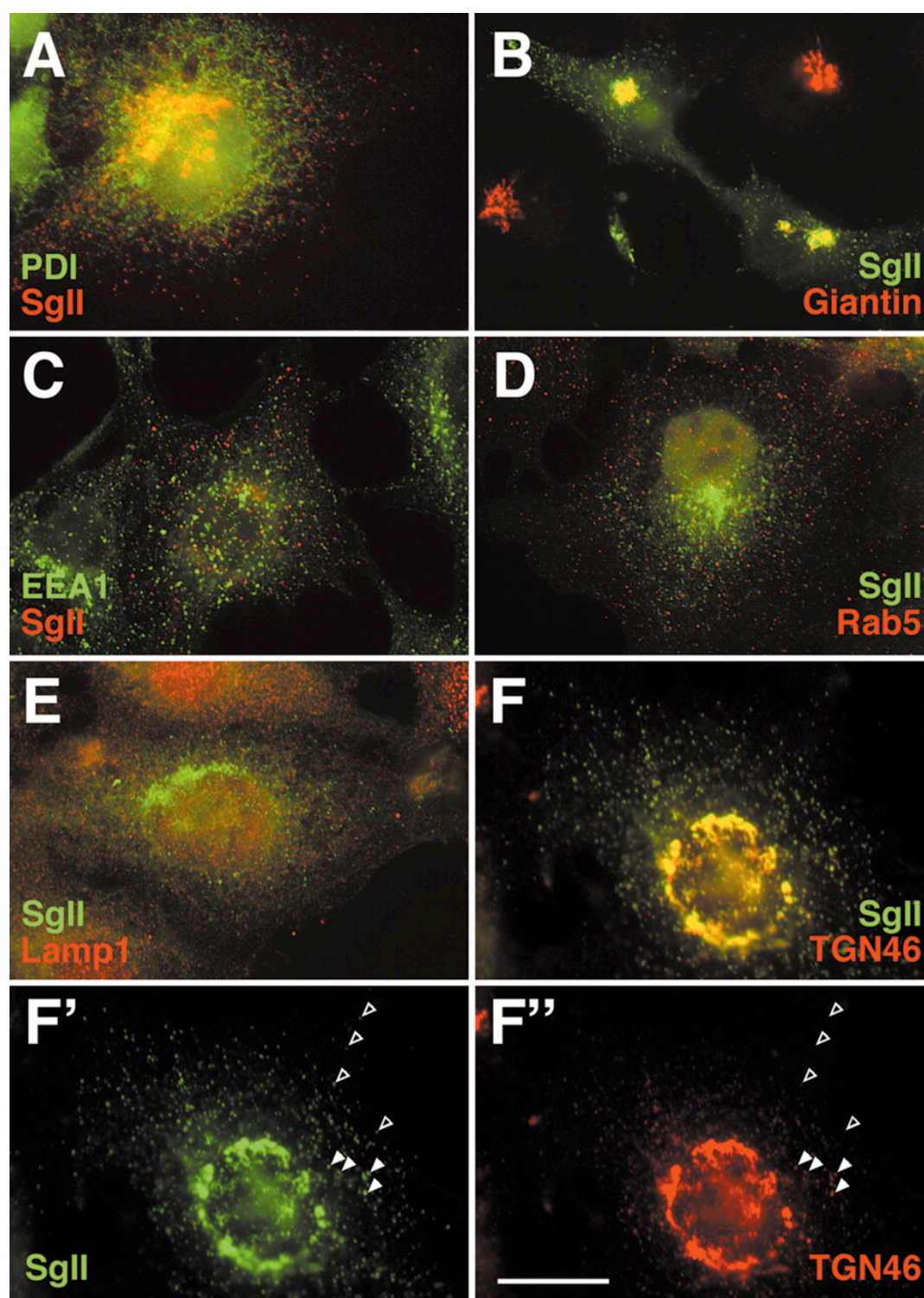
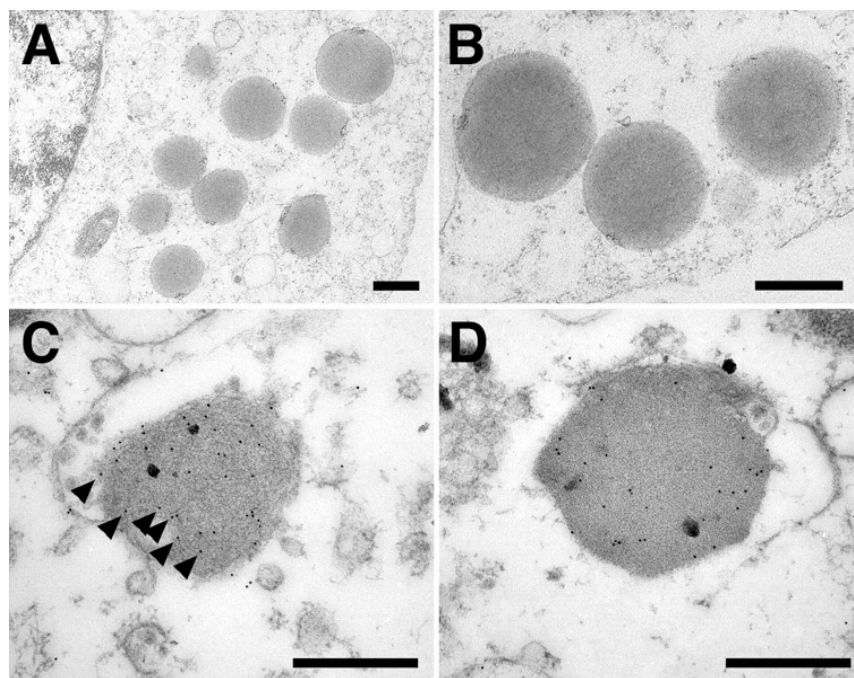


FIG. 12. Regulated cargo accumulates in post-Golgi structures.

Transfected COS-1 cells expressing SgII-myc (A and C) or SgII-C1 (B, D, E, and F) were processed for double-immunofluorescence microscopy staining for the myc/C1 epitope and for the organelle markers protein disulfide isomerase (PDI; ER), giantin (Golgi), EEA1 (early endosomes), rab5 (early and recycling endosomes), lamp-1 (late endosomes and lysosomes), or TGN46 (TGN), as indicated. The positions of some SgII-positive structures in panels F' and F'' which are positive or negative for TGN46 are pointed out by filled or open arrowheads, respectively. Bar, 20  $\mu$ m.

*Ultrastructural morphology of granule-like structures* — To facilitate the analysis by electron microscopy, COS-1 cells were transfected simultaneously with expression plasmids for the vasopressin precursor or SgII-myc, and for green fluorescent protein (GFP). The cells were then trypsinized and subjected to fluorescence-activated cell sorting to isolate the transfected, GFP producing cells. Upon processing of cells expressing pro-vasopressin for electron microscopy, structures of 0.4–1  $\mu\text{m}$  diameter were observed that were quite homogeneously filled with dense material and surrounded by a membrane (Fig. 13A–B) and were not detectable in untransfected COS-1 cells.

Granule-like structures formed by SgII-myc had a very similar ultrastructural appearance and an average size of 0.64  $\mu\text{m}$  ( $\pm 0.16 \mu\text{m}$ ;  $n=65$ ). Unlike our anti-neurophysin antibodies, the antibody against the myc epitope was suitable for immunogold electron microscopy. Anti-myc antibody in combination with a secondary antibody coupled to 10-nm gold particles clearly decorated the dense material of the granular structures demonstrating the presence of SgII-myc (Fig. 13C–D). The granule-like structures produced in COS-1 cells are similar in ultrastructural appearance to secretory granules observed in different endocrine tissues, but larger, since the latter are typically only 100–400 nm in diameter (Cross and Mercer, 1993).



**FIG. 13. Morphological analysis of granule-like structures by electron microscopy.**

COS-1 cells were transfected with expression plasmids encoding GFP and either pro-vasopressin (A and B) or SgII-myc (C and D). Fluorescent cells were selected by cell sorting and processed for electron microscopy. Thin sections shown in C and D were decorated with anti-myc antibodies and secondary antibodies coupled to 10-nm gold. Some gold particles are pointed out by arrowheads in panel C. The gold density on granule-like structures was 13 times higher than that in the cytosol (including the ER, which also contains SgII-myc). Bars, 0.5  $\mu\text{m}$ .

*Storage and stimulated secretion in COS-1 cells* — In pulse-chase experiments with transfected COS-1 cells, we have previously observed that approximately 85% of newly synthesized pro-vasopressin was secreted into the medium within 2 h (Beuret *et al.*, 1999), and within 4 h less than 10% was retained in the cells (data not shown). Sorting of pro-vasopressin into granule-like structures thus appeared rather inefficient, although it has to be considered that only half the cells expressing pro-vasopressin form accumulations. In Fig. 14, we determined the retention efficiency of SgII-C1 and CgB-C1, which form granule-like structures in all transfected cells. (C1-tagged proteins were analyzed, because they could be more efficiently immunoprecipitated than the myc-tagged versions.) SgII and CgB are naturally sulfated in the TGN and can be conveniently labeled with [<sup>35</sup>S]sulfate. As a constitutively secreted control protein, we used A1Pi<sup>TS</sup>, a version of A1Pi tagged at the C-terminus with the tyrosine sulfation sequence SAEDYEYPS (Leitinger *et al.*, 1994; Leitinger *et al.*, 1995; Kromer *et al.*, 1998).

Transfected COS-1 cells were pulse labeled with [<sup>35</sup>S]sulfate for 90 min at 19°C to accumulate sufficient amounts of labeled protein while retaining it in the TGN, and then chased at 37°C for up to 6 h. At different time points, the medium was removed and replaced by fresh medium. The labeled protein was immunoprecipitated from media and cells and analyzed (Fig. 14). A1Pi<sup>TS</sup> was almost completely secreted within the first hour of chase (lanes 1–7 and circles). In contrast, only 70% of CgB-C1 (lanes 8–13 and triangles) and less than half of SgII-C1 (lanes 14–19 and filled squares) was constitutively secreted within the first 2 h, and further release was slow. After 6 h, ~10% of CgB-C1 and ~40% of SgII-C1 were still retained intracellularly. The latter corresponds to a level of retention similar to that found for regulated secretory proteins in endocrine cell lines (e.g. exogenous CgB in PC12 cells) (Kromer *et al.*, 1998).

When the cells were labeled at 37°C for 1 h (Fig. 14, lanes 20–25 and open squares), ~40% of the labeled SgII-C1 was already secreted into the medium during the labeling period. After a 6-hour chase, ~25% was still stored intracellularly. Retention of SgII-C1 thus appears to be increased when labeling was performed at 19°C. This is most likely due to the increased concentration of the protein in the TGN when exit is blocked, and/or the extended residence time in the compartment where granule formation takes place.

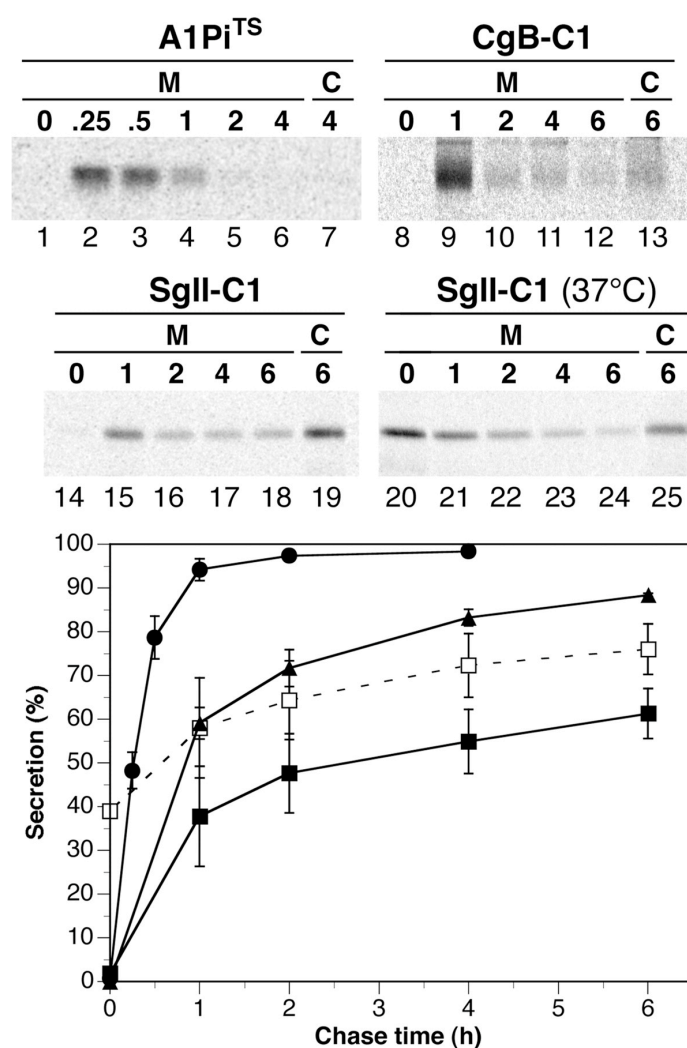


FIG. 14. Intracellular storage of CgB-C1 and SgII-C1 in COS-1 cells.

Kinetics of secretion of A1Pi<sup>TS</sup> (circles), CgB-C1 (triangles), and SgII-C1 (squares) were determined by [<sup>35</sup>S]sulfate labeling for 90 min at 19°C (lanes 1–19, and solid lines and filled symbols) or for 1 h at 37°C (lanes 20–25, and broken line and open symbols), followed by a chase at 37°C for up to 6 h. The medium was removed and replaced by chase medium at the indicated time points. Labeled protein in these media (M) and in the cells at the end of the experiment (C) was immunoprecipitated and analyzed by gel electrophoresis and autoradiography. The cumulative amounts of secreted protein is plotted in the graph in percent of the total (average with standard deviation of 3 or 4 experiments).



An obvious question is whether granule proteins stored in COS-1 cells can be stimulated to be released. To test this, cells were transfected with SgII-C1 (the regulated cargo that was most efficiently stored) were labeled for 90 min at 19°C with [<sup>35</sup>S]sulfate to load granules with radioactive SgII-C1, and chased for 3 h with excess unlabeled sulfate to allow the constitutively secreted protein to be released. The cells were then incubated with fresh medium with or without the calcium ionophore A23187 for 15 or 30 min. As shown in Fig. 15A, more than 10% of the initially labeled SgII-C1 was stimulated to be secreted by the ionophore after 15 min, and ~20% after 30 min. Based on the pulse-chase experiment of Fig. 14, this corresponds to ~20% and ~40%, respectively, of the retained pool after 3 h of chase. Stimulated release of SgII was accompanied by a significant decrease of the average density of granule-like structures by ~30% after 30 min incubation with A23187 (Fig. 15B;  $p < 0.0001$  according to Student's t-test). Secretion of SgII stored in granule-like structures in COS-1 cells can be induced by increased calcium concentration.

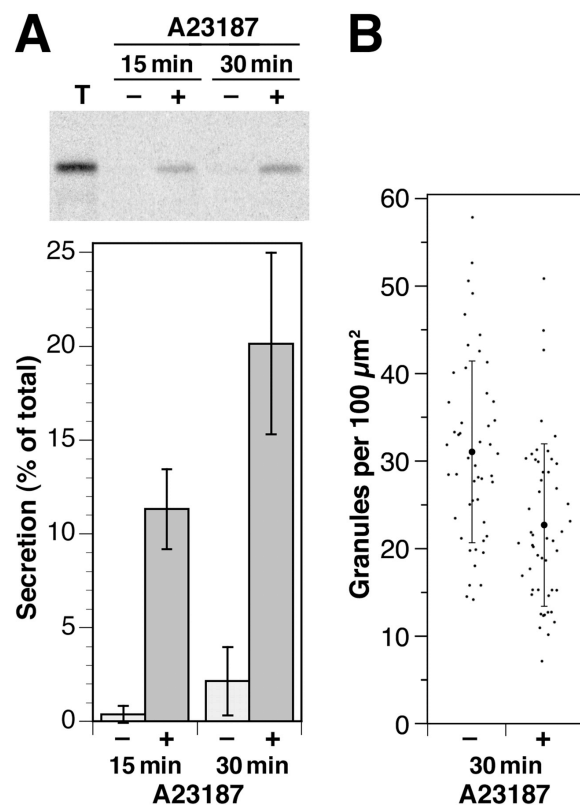


FIG. 15. **Stimulated secretion of SgII-C1 in COS-1 cells.**

(A) Transfected COS-1 cells were labeled with [<sup>35</sup>S]sulfate for 90 min at 19°C and chased for 3 h at 37°C with excess nonradioactive sulfate. The medium was then replaced by fresh medium with or without 1  $\mu\text{M}$  A23187 for 15 or 30 min. Labeled SgII-C1 secreted during this time was analyzed by gel electrophoresis and autoradiography in parallel with a sample representing total SgII-C1 immunoprecipitated immediately after labeling (T). The amounts of protein secreted in the absence or presence of stimulant are plotted in the graph in percent of total labeled SgII-C1. The average with standard deviation of 5 determinations is shown. (B) Transfected COS-1 cells were incubated for 30 min with fresh medium with or without 1  $\mu\text{M}$  A23187 and then fixed and processed for immunofluorescence. The distribution of densities of granule-like structures of 50 cells, the average and standard deviation are plotted for each condition. The density distribution is significantly different according to Student's t-test ( $p < 0.0001$ ).

## Discussion

Secretory granules of endocrine and neuroendocrine cells typically contain hormone precursors and granins as their main cargo. We have made the observation that the hormone precursors pro-vasopressin, pro-oxytocin, and POMC, as well as the granins SgII and CgB are able to generate granule-like structures when expressed in cell lines lacking a regulated secretory pathway. The observed structures resemble secretory granules with respect to several criteria: They contain the protein at high concentrations as judged by immunofluorescence, and in densely packed form, as is apparent in differential interference contrast microscopy. They are membrane-bounded structures of similar appearance in electron microscopy to that of certain endocrine granules in tissues, although larger. They represent post-Golgi structures, devoid of markers of endosomes or lysosomes, but containing low amounts of TGN46. These structures are not formed by a constitutively secreted protein. When coexpressed with SgII, A1Pi is not found in the granule-like structures formed by SgII-myc, which demonstrates sorting between constitutive and regulated cargo. The formation of SgII accumulations is accompanied by significant intracellular storage as observed in pulse-chase experiments. Incubation with the calcium ionophore A23187 induces secretion of stored SgII.

However, the similarity of granule-like structures in COS-1 cells with secretory granules in endocrine cells has its limitations. Storage efficiency, although quite high for SgII (Fig. 14), was lower for CgB, and hardly measurable for pro-vasopressin (Beuret *et al.*, 1999). The structures were 2–3 times larger, for pro-vasopressin even more, than natural endocrine secretory granules. The precursor proteins were not processed, because the appropriate enzymes are not expressed. Continued release of SgII-C1 upon stimulation with A23187 during 30 min (Fig. 15) might suggest inefficient docking to the plasma membrane, slow membrane fusion, or slow transport to the periphery of the cell. It is likely that endocrine cells have specialized mechanisms to regulate these processes and to optimize their efficiency. The effect of calcium ionophore on SgII secretion may also be related to calcium-induced exocytosis of membranes previously observed in CHO and 3T3 cells in electrophysiological studies (Coorssen *et al.*, 1996; Ninomiya *et al.*, 1996), a process that seems to play a role in plasma membrane enlargement in differentiation and membrane repair (Borgonovo *et al.*, 2002).

CgB has previously been expressed in Vero cells as a GFP fusion protein and visualized in punctate structures (Wacker *et al.*, 1997; Rustom *et al.*, 2002). They were interpreted as constitutive secretory vesicles, because ~80% of labeled CgB-GFP was secreted within 2 h (Wacker *et al.*, 1997). After submission of our study, CgB was shown to generate granular structures when expressed in COS-7 and NIH3T3 cells as visualized by electron microscopy (Huh *et al.*, 2003), in agreement with our own findings.

Our results indicate that expression of regulated cargo proteins is sufficient to perform the initial stages of regulated secretion in the background of non-endocrine cells. The simplest explanation is that granule formation is the result of self-organizing properties of regulated cargo, mainly via their ability to selectively aggregate under TGN conditions of high calcium and low pH (Chanat and Huttner, 1991; Colomer *et al.*, 1996) and potentially to interact with

the TGN membrane (as reported for CgB) (Pimplikar and Huttner, 1992). The forming granule may spontaneously detach from the TGN as a result of the dynamics of fusion and fission of the network membranes. Contaminating material destined for endosomes/lysosomes or for the cell surface (such as mannose-6-phosphate receptors or TGN46) is removed by clathrin-coated vesicles and constitutive-like secretory vesicles, respectively, just as it is from the original TGN membrane. This corresponds to the sorting-by-retention model and in the simplest case may not require any additional machinery specific for the regulated pathway up to this point. The situation may be similar to that of von Willebrand factor precursor, a multimeric protein of endothelial cells and megakaryocytes involved in platelet adhesion. Upon expression in monkey kidney CV-1 cells, structures were observed that were morphologically similar to the endothelial-specific Weibel-Palade bodies (Voorberg *et al.*, 1993).

Whatever machineries are involved in generating granules as observed in COS-1 cells, they are either constitutively expressed also in non-endocrine cells or they are induced upon expression of regulated cargo. The first possibility is supported by a study by Chavez *et al.* (Chavez *et al.*, 1996) which suggested that constitutive secretory cells such as CHO or L cells have a cryptic regulated pathway. A significant fraction of [<sup>35</sup>S]sulfate-labeled free glycosaminoglycan chains were stored intracellularly and could be stimulated to be secreted by phorbol ester or by increased cytoplasmic calcium.

Supporting the second possibility, CgA has been proposed to play the role of the master control molecule of initiation and regulation of dense-core secretory granule biogenesis (Kim *et al.*, 2001), even in CV-1 cells. The interpretation of CgA as a on/off switch of granule formation has raised controversy (Day and Gorr, 2003; Kim *et al.*, 2003), and CgA has been alternatively proposed to act as one of several assembly factors in granule biogenesis (Day and Gorr, 2003). Our results indeed show that CgA is not unique in its ability to induce the formation of granules. All five regulated cargo proteins tested in our study induced granule-like structures in COS-1 cells, although with different efficiencies. Only a fraction of the cells expressing pro-vasopressin, pro-oxytocin, or POMC generated granules, whereas virtually all cells expressing SgII or CgB did so. This supports the proposed functions of granins as assembly factors or helpers of granule formation and as carrier proteins. Similarly, it had been observed that overexpression of CgB increased granule sorting and processing of POMC in AtT-20 cells (Natori and Huttner, 1996). The peptide hormone precursors share the ability to assemble into granule-like structures by themselves, although to a lesser extent than the granins. Auxiliary granins are certainly not the only factors lacking in COS-1 cells that increase efficiency of sorting or other steps in regulated secretion in professional regulated secretory cells. The analysis of granule formation and function in nonendocrine cells may be useful to identify such factors and their mechanisms of action.



## CHAPTER II

### **Characterization of Granule-like Structures in Non-Endocrine Cells**

Manuscript

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## Abstract

Regulated secretory proteins have the ability to induce granule-like structures in non-endocrine cells. Helper proteins, the granins, are more efficient than prohormones and induce granule-like structures in all expressing cells. Upon coexpression with prohormones, granins modulate morphology and frequency of induced structures. Stable expression revealed that the number of granule-like structures positive for a hormone cargo is significantly increased when secretogranin II is coexpressed. Pro-vasopressin and secretogranin II sorted together into newly generated structures derived from trans-Golgi network membranes. The granule-associated proprotein convertase (PC) PC3 and PC6A entered granule-like structures induced by secretogranin II, however, PC3 and PC6A were also observed in separate vesicles containing no secretogranin II. In contrast, PC2 was not found in SgII-induced granules and did not induce granule-like structures. In addition, granule-like structures induced by secretogranin II recruited the inositol-3-phosphate receptor supporting a role for secretogranin II in calcium mobilization. Storage of secretogranin II varied depending on cell type from hardly detectable to more than 70% of stored secretogranin II after 6 h of chase. Our findings suggest that non-endocrine cells can form minimal secretory granules in the absence of an endocrine-specific machinery. However, additional mechanisms are required for efficient sorting of accessory proteins and intracellular storage.

## Introduction

The regulated secretory pathway is exclusively found in exocrine, endocrine, and neuroendocrine cells where it serves for synthesis and storage of hormones and enzymes for instantaneous release upon extracellular stimulation. In the trans-Golgi network (TGN), proteins destined for regulated secretion are sorted away from constitutively secreted proteins and enter secretory granules where they are stored in a densely aggregated form. It remains unclear how secretory granules are formed. The sorting-for-entry model suggests a receptor-mediated sorting process (Kelly, 1987) similar to the mannose-6-phosphate-dependent transport of lysosomal enzymes (Ghosh *et al.*, 2003). Regulated secretory proteins, or an aggregate thereof, bind to a specific sorting receptor which allows entry into a secretory granule. Proteins that fail to interact with a receptor follow the default pathway for constitutive secretion. The sorting-by-retention model proposes the formation of an initial immature secretory granule (Arvan and Castle, 1987). pH- and  $\text{Ca}^{2+}$ -dependent selective aggregation of regulated secretory proteins starts in the TGN where immature secretory granules - including constitutive and regulated secretory proteins - bud by an as yet unknown mechanism. Sorting occurs during the maturation process when captured contaminant proteins are removed from immature secretory granules by clathrin coated vesicles (Dittie *et al.*, 1997; Klumperman *et al.*, 1998). However, there is also evidence for selective sorting into the constitutive secretory pathway. A recent study showed that a truncated version of the Golgi resident protein Cab45 is secreted without entering immature secretory granules suggesting that its association with Golgi and/or post-Golgi membranes serves as a means for selective sorting into the constitutive secretory pathway (Lara-Lemus *et al.*, 2006).

Over the past years, it has been shown that at least the initial steps of secretory granule formation can also be performed in non-specialized cell types. The granin family members chromogranin A (CgA), chromogranin B (CgB), and secretogranin II (SgII) were shown to be involved in this process. Overexpression of CgA in the non-endocrine fibroblast cell line CV-1 led to the generation of large dense-core granules (Kim *et al.*, 2001). CgA was even suggested to control secretory granule formation acting as an on/off switch of a gene or assembly program. The key regulatory role of CgA was called into question by the finding that CgB proved to be even more effective than CgA in inducing granule-like structures in COS-7 and NIH3T3 fibroblasts (Huh *et al.*, 2003). In another study, we showed that the hormone precursors pro-vasopressin, pro-oxytocin, and POMC, as well as the granins CgB and SgII are capable to induce granule-like structures in various non-endocrine cells lines such as HepG2, 3T3, MDCK, CHO, HEK293, and COS-1 (Beuret *et al.*, 2004). The available data suggest that a cryptic assembly program is launched in non-endocrine cells to package and store regulated secretory proteins. The basic mechanism for the formation of secretory granules can be induced in fibroblast-like and endothelial cells. However, the definition and characterization of granule-like structures has caused controversy. The role of CgA was reinvestigated by Malosio *et al.* (2004). CgA sorted to discrete organelles of CV-1 cells were interpreted as post-TGN compartments destined for degradation in lysosomes. A large fraction of transfected CgA colocalized with the lysosomal/late endosomal markers Lamp-1 and Lamp-3. In contrast, we found that granule-like structures induced by SgII (Beuret *et al.*,

2004) and pro-vasopressin (not shown) did not colocalize with the lysosomal marker Lamp-1 in COS-1 cells.

In this study, we analyzed the efficiency of different regulated cargo proteins to induce granule-like structures of different morphologies and quantified these structures. We tested the role of SgII as a helper for packaging the hormone precursor pro-vasopressin. Intracellular storage of SgII was assessed in different non-endocrine cell lines. Further, we studied the sorting behavior of accessory proteins including the proprotein convertases PC2, PC3, PC6A, and PC7 as well as the IP3-R/ $\text{Ca}^{2+}$  channel.



## Materials and Methods

*cDNA Constructs* — cDNAs for human SgII and CgB were gifts from H. Gerdes, University of Heidelberg, for human pro-vasopressin from M. Ito, Northwestern University, Chicago, IL, for porcine POMC from G. Boileau, University of Montreal, and for human oxytocin from J. Amico, University of Pittsburg. The cDNAs encoding mouse PC2, human PC3, mouse PC6A, and rat PC7 were gifts from W. van de Ven (Flanders Institute of Biotechnology, Leuven, Belgium). To introduce a C-terminal C1-epitope (14 C-terminal codons of the asialoglycoprotein receptor H1) (Beuret *et al.*, 2004) a HindIII site was introduced by PCR in front of the sequence encoding PC2 using the mutagenic primer CGCAAGCTTCTCACTATAGGGAGACC (HindIII restriction site is underlined) and the stop coding was replaced with an Asp718 site using the primer GCGGGTACCGTTCTTTCTCAGGATACTTTG (Asp718 site is underlined) and fused in-frame to the C1-epitope. Similarly, PC6A was amplified with the primers CGCAAGCTTCCGGACTCAGATCTCG (HindIII site is underlined) and GCGGGTACCGCCTTGGAATGTACATGTT (Asp718 site is underlined), and PC7 was amplified with the primers CGCAAGCTTCCGGACTCAGATCTCG (HindIII site is underlined) and GCGGGTACCGCAGATCTGTCCATCC (Asp718 site is underlined) for fusion to a C1-epitope at the C-terminus (Beuret *et al.*, 2004). The generation of PC3 constructs were previously described (Stettler *et al.*, 2005). To generate SgII-myc and SgII-C1 constructs, a myc or C1-epitope was fused to SgII via a KpnI linker. The cDNAs were subcloned into the expression vectors pECE, pCB6 or pCDNA5/FRT/TO (Invitrogen).

*Cell Culture and Transfection* — Human embryonic kidney (HEK) 293 T-REx cells (Invitrogen) were cultured in Dulbecco's minimal essential medium (DMEM) supplemented with 2 mM L-glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin, 4 µg/ml blasticidin, 100 µg/ml zeocin, and 10% fetal calf serum at 37 °C with 5% CO<sub>2</sub>.

Cells were transfected using Lipofectin (Invitrogen) with 12 µg of pCB6 plasmid DNA/10-cm dish at 40% confluency. After 48 h, cells were incubated with 1 µg/ml G418 for clone selection. pCDNA5/FRT/TO plasmids were transfected using a CaPO<sub>4</sub>-precipitation transfection protocol. For a 10-cm dish at 60% confluency 10 µg of plasmid DNA and 50 µg of helper plasmid pOG44 (Invitrogen) in 0.5 ml 4 mM CaCl<sub>2</sub> were vortexed with 0.5 ml 2x HBS (280 mM NaCl, 1.5 mM Na<sub>2</sub>HPO<sub>4</sub>, 50 mM HEPES, pH 7.05) at room temperature and immediately added to the cells in 5 ml fresh medium without serum. After 5 h, cells were supplemented with 5 ml normal growth medium (without blasticidin and zeocin), and after 48 h incubated with 180 µg/ml hygromycin B and 4 µg/ml blasticidin in the absence of zeocin for clone selection. Resistant cell lines were analyzed by immunofluorescence microscopy, Western blotting and β-galactosidase activity. For induction cells were incubated with 1 µg/ml tetracycline for 24 h unless indicated otherwise.

COS-1 and HeLa cells were cultured in DMEM supplemented with 2 mM L-glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin, and 10% fetal calf serum at 37 °C with 7.5% CO<sub>2</sub>. Cells were transfected with Lipofectin (Invitrogen) with 2 µg of plasmid DNA/35-mm dish at 40% confluency and processed 48 h after transfection.

*Immunofluorescence and Immunoblotting Procedures* — Antisera used for immunofluorescence were rabbit anti-neurophysin II (NPII) for pro-vasopressin and oxytocin, rabbit anti-human adrenocorticotrophic hormone (ACTH) for POMC (Sigma), rabbit anti-A1Pi from Jerry L. Brown (University of Colorado Health Sciences Center, Denver, CO), monoclonal mouse anti-myc (9E10) (Evan *et al.*, 1985), rabbit anti-C1 (Geffen *et al.*, 1993). Antibodies were used at a dilution of 1:100, except anti-ACTH (1:200). As secondary antibodies non-crossreacting Cy2-labeled goat anti-rabbit and Cy3-labeled goat anti-mouse were used. For immunoblotting a rabbit anti-secretoneurin antiserum (a gift from R. Fischer-Colbrie, University of Innsbruck) was used at a dilution of 1:1000. Cells were fixed and stained as previously described (Beuret *et al.*, 2004).

*Electron Microscopy* — Affinity-purified polyclonal rabbit anti-IP3-R type I antibodies were a gift from Seung Hyun Yoo (Inha University College of Medicine, Incheon, Korea). SgII was stained with a mouse anti-myc antiserum or with rabbit anti-secretoneurin (a gift from R. Fischer-Colbrie, University of Innsbruck). Pro-vasopressin was stained with rabbit anti-NPII. The secondary antibodies used were anti-rabbit coupled to 5-nm gold and anti-mouse coupled to 10-nm gold particles or anti-rabbit coupled to 10-nm gold. Control experiments were performed to confirm that secondary antibodies did not crossreact.

Cells were fixed in 3% formaldehyde and 0.2% glutaraldehyde overnight at 4 °C, washed three times with 0.1 M phosphate buffer, pH 7.4, and incubated with 50 mM NH<sub>4</sub>Cl for 30 min at room temperature. After washing three times with phosphate buffer, cells were scraped, mixed with 10% gelatin and processed for ultrathin cryosectioning as described by (Tokuyasu, 1989; Liou *et al.*, 1996). Small pieces of gelatin were infiltrated on 1.725 M sucrose and PVP solution overnight at 4°C, mounted on cutting pins, frozen in liquid nitrogen and cryosectioned at -125°C using a Leica Ultracut UCT ultramicrotome. Sections were picked up with 1:1 2% methylcellulose and 2.3 M sucrose and transferred to nickel grids.

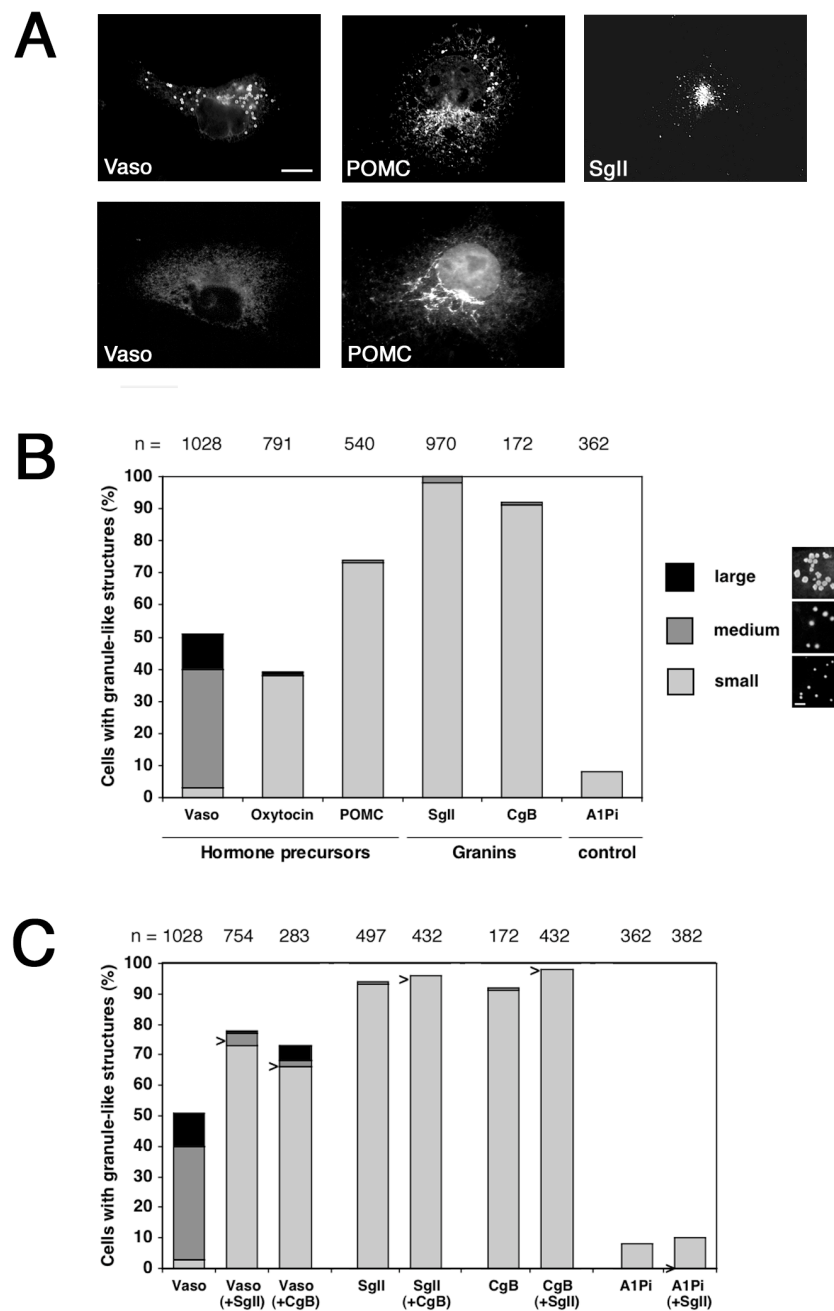
For immunogold labeling cells were incubated with appropriate primary antibodies for 1-4 h and colloidal gold-conjugated secondary antibodies in PBS containing 2% BSA for 1 h. Grids were fixed in glutaraldehyde, washed in ddH<sub>2</sub>O, stained in 0.4% uranylacetate and 2% methylcellulose for 10 min and viewed with a Philips CM100 electron microscope.

*Storage Assay* — To analyze the secretion behavior, COS-1 and HeLa cells transfected with SgII-C1 or A1Pi<sup>TS</sup> were starved for 30 min in sulfate-free medium, and pulse-labeled with [<sup>35</sup>S]sulfate in starvation medium for 60 min at 37 °C followed by a chase of 2 or 6 h in normal growth medium. Stable HEK293 cells were labeled with [<sup>35</sup>S]methionine for 30 min at 37°C and chased for 6 h. Labeled protein secreted during that time as well as the fraction retained in the cells were immunoprecipitated, analyzed by SDS-PAGE and autoradiography, and quantified by PhosphorImager.

## Results

*Granins are more efficient than prohormones in inducing granule-like structures* — Besides hormones, the main physiological content, there are additional major cargo proteins sorted into secretory granules. Major constituents are the granins, a family of acidic,  $\text{Ca}^{2+}$ -binding proteins that have been suggested to function as helper proteins for the packaging of hormones into secretory granules (Rosa *et al.*, 1985; Huttner *et al.*, 1991). We reported that prohormones and granins are capable to induce granule-like structures when expressed in non-endocrine cells (Beuret *et al.*, 2004). We quantified the efficiency of granule-like structure formation and found that granins are more efficient than prohormones. Virtually all COS-1 cells transfected with SgII or CgB produced granule-like structures (Fig. 16A and B). The prohormones for vasopressin, oxytocin, and POMC induced granule-like structures in 51%, 39%, and 75% of transfected COS-1 cells, respectively, while the remaining cells showed staining of the ER and Golgi only. A1Pi was used as control for a constitutively secreted protein. As expected, 92% of A1Pi-transfected cells showed a weak staining of the ER and Golgi, while only around 8% of cells showed accumulations. This value is markedly lower than what we obtained for regulated secretory proteins and therefore considered as background level. When pro-vasopressin was coexpressed with SgII or CgB, the number of cells positive for pro-vasopressin structures increased from 51% to 78% or 72%, respectively (Fig. 16C). This implies that granins assist in triggering the formation pro-vasopressin containing granule-like structures.

Granule-like structures induced by pro-vasopressin were exceptional in that they had different sizes often with diameters of up to several micrometers. We qualitatively classified granule-like structures as small ( $<1\ \mu\text{m}$ ), medium-sized ( $1\text{--}3\ \mu\text{m}$ ), or large ( $3\text{--}4\ \mu\text{m}$ ) as shown in Fig. 16B. The quantifications showed that 23% of pro-vasopressin expressing cells had large structures, 70% were medium-sized while only 7% were of the small type. In contrast, cells transfected with other prohormones or granins mainly produced structures in the fine sub-micrometer size range. Interestingly, coexpression of pro-vasopressin with SgII or CgB resulted in a change of morphology. All granule-like structures positive for both cargo proteins appeared in the fine sub-micrometer size range. These observations support the proposed role of granins as helper proteins (Rosa *et al.*, 1985). Granins modulate the efficiency of formation and have an effect on the morphology of pro-vasopressin containing granule-like structures.

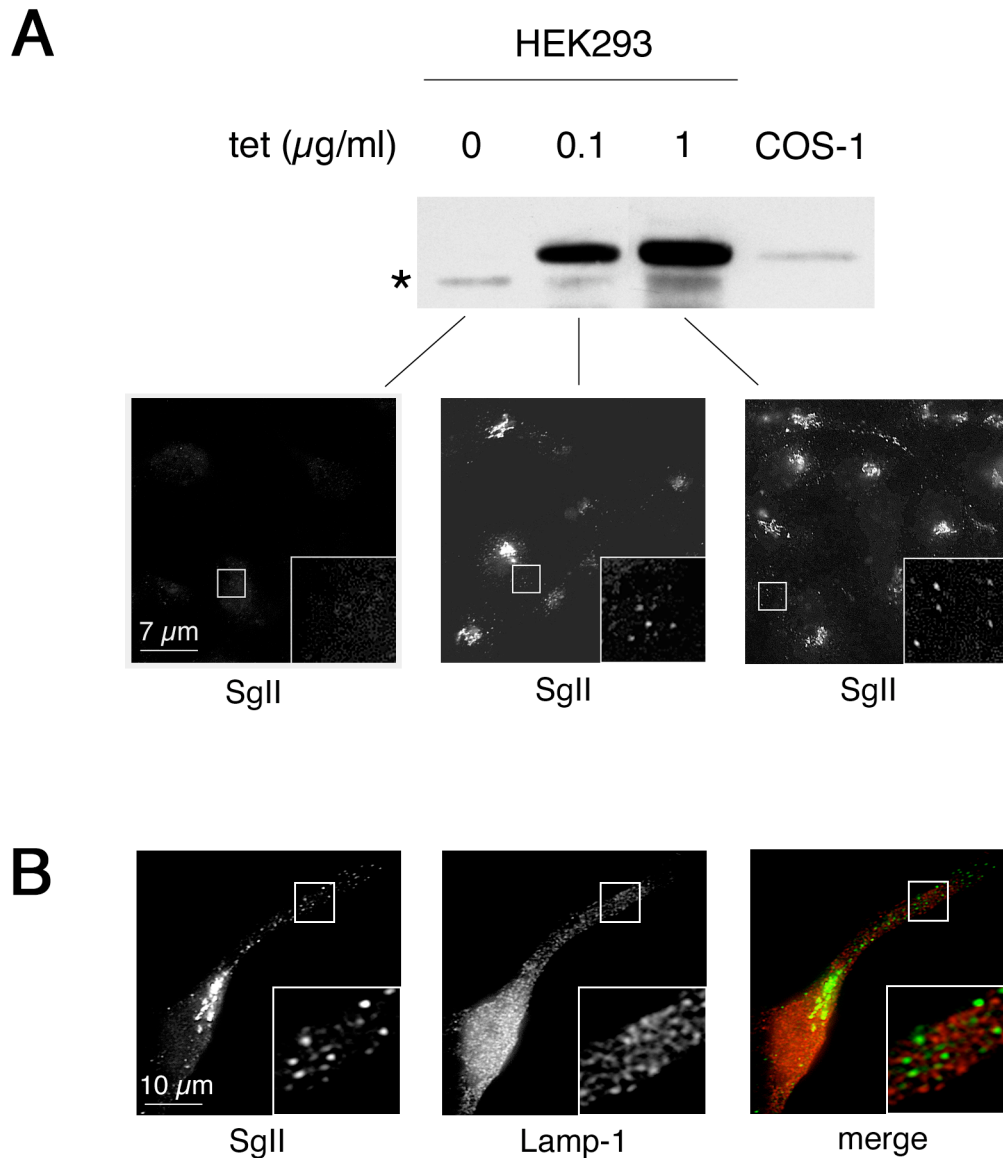


**FIG. 16. Granins modulate frequency and size of granule-like structures upon co-expression with other regulated cargo proteins in COS-1 cells.**

A: Transfected COS-1 cells expressing pro-vasopressin (Vaso), POMC, and SgII-myc were stained with antibodies against NPII, ACTH, and the myc-epitope, respectively. In the upper panel, cells with granule-like structures are shown while the cells in the lower panel show reticular or Golgi staining. B: Granule-like structures induced by pro-vasopressin, pro-oxytocin, POMC, SgII, and CgB were quantified and classified with respect to morphological appearance. The constitutively secreted protease inhibitor A1Pi was used as a control. C: The granins SgII-myc (or -C1) and CgB-C1 (or -myc) were coexpressed with pro-vasopressin and assessed for efficiency of formation and morphological appearance of granule-like structures. ">" indicates the percentage of cells in which at least a sub-population of the granule-like structures were stained for both proteins. Bars, 20  $\mu$ m for A, 5  $\mu$ m for B.

*Granule-like structures in an inducible cell system* — The transient system reaches its limits when two cargo proteins are expressed at a time. Since only a small fraction of cells are cotransfected and expression levels can vary between individual cells, we decided to use a stable expression system. Stable cell lines are more controlled since all cells derived from a single clone are genetically identical and have similar expression levels. We established stable cell lines expressing SgII-myc under the control of a tetracycline-inducible promoter in HEK293 Flp-In T-REx cells. When expression was induced with tetracycline, all cells of the stable clone S18 generated granule-like structures similar to the ones observed in transiently transfected COS-1 cells (Beuret *et al.*, 2004). Induction with 1  $\mu\text{g/ml}$  tetracycline resulted in the appearance of more granule-like structures than induction with a lower dose of 0.1  $\mu\text{g/ml}$  tetracycline (Fig. 17A). Similarly, the signal detected by Western blot was dependent on the tetracycline dose confirming that expression of SgII-myc can be tuned, and that this leads to a dose-dependent increase or decrease of granule-like structure formation.

CgA stably expressed in CV-1 cells was shown to colocalize with the late endosomal/lysosomal markers Lamp1 and Lamp3 (Malosio *et al.*, 2004). For this reason we tested our stable cell lines for colocalization with the endosomal/lysosomal marker Lamp-1 (Fig. 17B). However, SgII did not colocalize with Lamp-1 which makes it unlikely that the observed structures represent accumulations in transit to lysosomes for degradation.



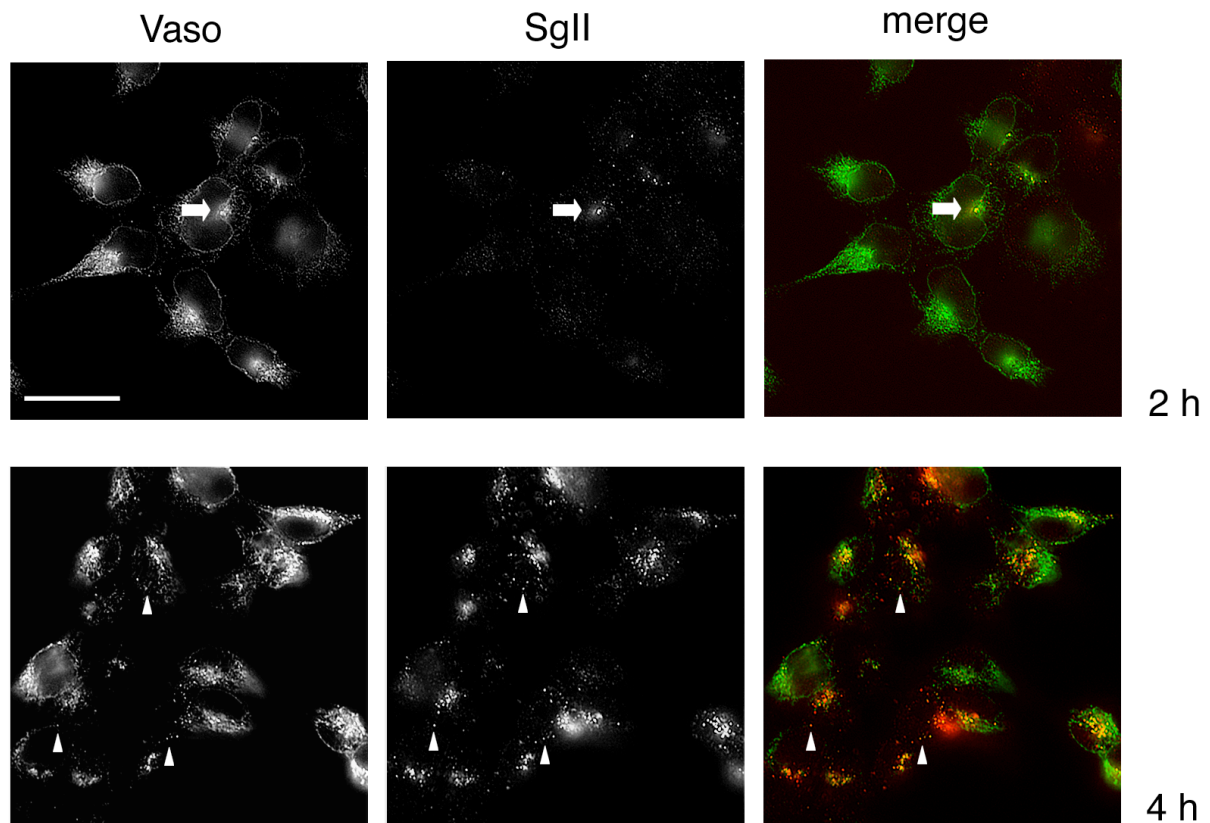
**FIG. 17. SgII expression leads to the formation of granule-like structures devoid of the lysosomal marker Lamp-1.**

A: Stable HEK S18 cells were induced for 24 h with a final concentration of 0, 0.1 or 1  $\mu\text{g/ml}$  tetracycline (tet). Standardized amounts of cell extract were analyzed by Western blot using an antibody against secretoneurin. In parallel, transiently transfected COS-1 cells were analyzed. The asterisk (\*) indicates a background band. For immunofluorescence analysis, SgII was stained with an antibody against the myc-epitope. B: Stable HEK S18 cells were induced for 24 h with 1  $\mu\text{g/ml}$  tetracycline and costained with antibodies against the myc-epitope (green) and against endogenous Lamp-1 (red).

*Sorting of stably expressed pro-vasopressin is enhanced by SgII* — To study the effect of granin expression on the sorting behavior of hormones we established stable cell lines coexpressing pro-vasopressin and SgII-myc. The cDNA encoding pro-vasopressin was inserted by conventional random integration into the genome of HEK293 Flp-In T-REx cells. The cDNA encoding SgII-myc was fused to the tetracycline-response element of pCDNA5/FRT/TO and integrated into a pro-vasopressin-expressing clone by site-specific recombination with help of the flip recombinase as described by the manufacturer. Stable clones were isolated with help of antibiotic selection markers and tested for pro-vasopressin expression and regulable SgII-myc expression.

Stable cell lines expressing pro-vasopressin (in the absence of SgII-myc) were analyzed for formation of granule-like structures by immunofluorescence studies. Out of fifteen clones positive for expression, six had granule-like structures in the majority of cells while five had granule-like structures in only about 10% of cells. The remaining four clones had no peripheral accumulations at all but stained for ER, Golgi and TGN. Unlike in transiently transfected cells, where granule-like structures induced by pro-vasopressin were often particularly big, no large structures were observed in stable cell lines. The structures induced by pro-vasopressin or SgII-myc had a similar size. A reason for this could be that big structures are cytotoxic, and therefore cells generating big structures died during the antibiotic selection procedure.

The stable cell clone V55S34 expressing pro-vasopressin continuously and SgII-myc inducibly, was incubated with tetracycline for 2-4 h (Fig. 18). The appearance of structures containing SgII-myc was first observed after 2 h in the Golgi region where it colocalized with pro-vasopressin. After 4 h, the majority of cells showed peripheral granule-like structures that were positive for SgII-myc and pro-vasopressin. The data show that cargo proteins are cosegregated into newly generated structures emerging from the Golgi.

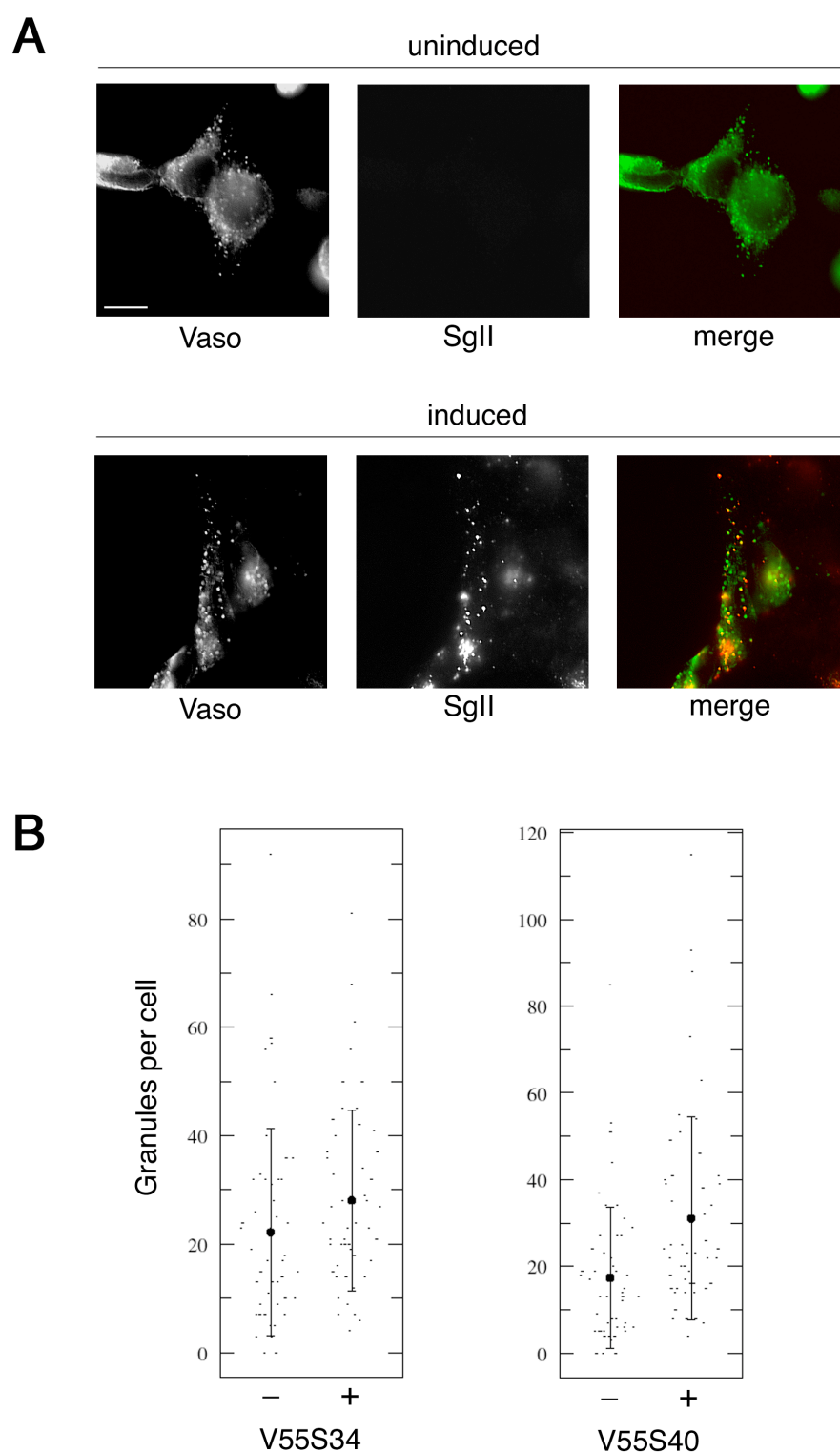


**FIG. 18. SgII and pro-vasopressin cosort into newly generated structures that first appear in the Golgi region.**

Stable HEK293 T-REx cells (clone V55S34) expressing pro-vasopressin continuously and SgII-myc under the control of a tetracycline-responsive promoter were incubated with tetracycline for 2 or 4 h, as indicated. Staining was performed with antibodies against NPII (green) and against the myc-epitope (red). The arrows indicate the first appearance of SgII in the Golgi region. The arrowheads indicate granule-like structures that are positive for both cargo proteins. Bar, 20  $\mu$ m.

*SgII helps to generate pro-vasopressin containing granule-like structures* — Clones V55S34 and V55S40 (expressing continuously pro-vasopressin and inducibly SgII-myc) were further analyzed for effects caused by the induction of SgII-myc. The number of granule-like structures positive for pro-vasopressin was used as a measure to assess the helper effect of granins. When expression of SgII-myc was induced for 24 h newly generated structures stained positive for SgII-myc as well as for pro-vasopressin (Fig. 19A). The number of pro-vasopressin-positive structures per cell significantly increased from 22 to 28 in clone V55S34 and from 17 to 31 in clone V55S40 (Fig. 19B;  $p < 0.001$  according to Student's t-test). In conclusion, SgII-myc induction had a significant effect on the generation of pro-vasopressin containing structures, and these observations are in agreement with the proposed role of granins as helper proteins for sorting hormones into secretory granules.



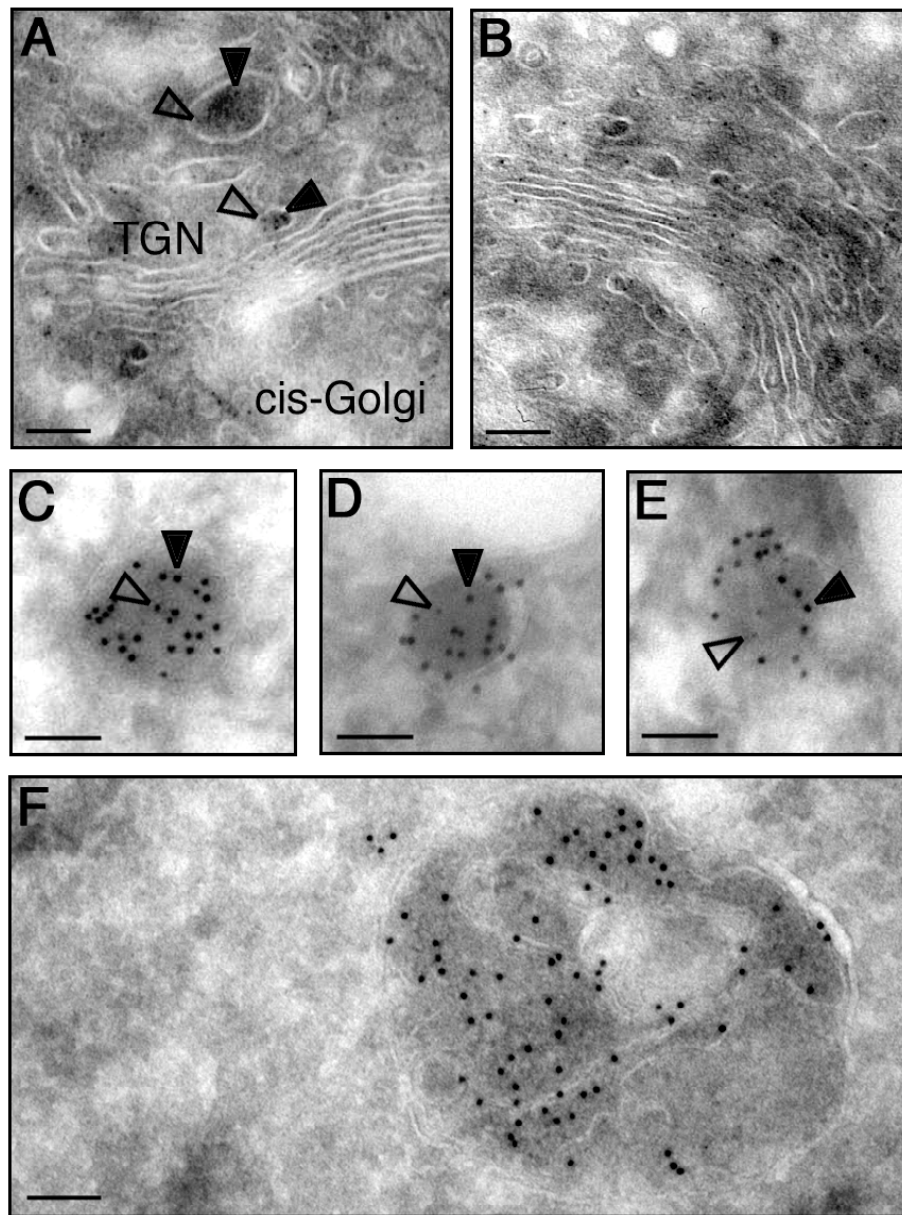


**FIG. 19. The number of pro-vasopressin-positive structures is increased upon expressing SgII.**

A: Stable HEK V55S34 cells expressing pro-vasopressin and SgII-myc were incubated with tetracycline for 24 h (lower panel) and stained with antisera against NPII and the myc-epitope. Uninduced cells were analyzed in parallel (upper panel). B: Uninduced (-) and induced (+) stable cell lines V55S34 and V55S40 were analyzed for the formation of granule-like structures. In 50 randomly picked cells, granule-like structures positive for pro-vasopressin were quantified. The total number of structures in each cell, the average and standard deviation are plotted for both conditions. The numbers are significantly different according to Student's t-test ( $p < 0.11$  for V55S34,  $p < 0.001$  for V55S40). Bar, 10  $\mu\text{m}$ .

*Granule-like structures form at the TGN* — Clone V55S34 was subjected to electron microscopic analysis. Cells were induced for 5 h before treatment. Electron micrographs showed granule-like structures emerging at the TGN (Fig. 20A-B). SgII and pro-vasopressin were detected in the Golgi stacks and colocalized in single membrane-bound vesicles which had a diameter of approximately 100-200 nm (Fig. 20C-E). The data show that SgII and pro-vasopressin are cosorted into newly formed membrane vesicles. Electron microscopic data are in agreement with the immunofluorescence analysis that also showed the appearance of granule-like structures in the Golgi region (Fig. 18).

Immunogold labeled pro-vasopressin was occasionally also detected in multivesicular bodies (Fig. 20F). Although we previously observed that high intracellular  $\text{Ca}^{2+}$  could stimulate the release of some stored SgII, spontaneous fusion of granule-like structures may not be the only mechanism of elimination. Eventual lysosomal delivery (e.g. by autophagy) might also contribute to the removal in stable cell lines. Immunofluorescence analysis of COS-1 cells (Beuret *et al.*, 2004) and stable HEK S18 cells (Fig. 17B) showed that SgII did not colocalize with the lysosomal marker Lamp-1 which argues against lysosomal delivery of SgII. However, occasional colocalization of pro-vasopressin with Lamp-1 was observed (data not shown).



**FIG. 20. Secretory granule formation starts in the TGN where the cargo proteins pro-vasopressin and SgII cosort into nascent vesicles.**

The electron micrographs represent immunogold labeled cryosections of stable HEK V55S34 cells that had been induced with tetracycline for 5 h. The arrowheads indicate pro-vasopressin (5 nm, empty arrowheads) and SgII-myc (10 nm, filled arrowheads) colocalizing in membrane-bound dense-core vesicles. For pictures A and B, cells were sequentially stained with rabbit anti-NP11, 5-nm gold coupled anti-rabbit antiserum, rabbit anti-secretoneurin, and 10-nm gold coupled anti-rabbit antiserum. For pictures C-E, cells were stained with rabbit anti-NP11 and mouse anti-myc primary antibodies before incubation with 5-nm gold coupled anti-rabbit and 10-nm gold-coupled anti-mouse secondary antibodies. For picture F, sections were stained with anti-NP11 and 10-nm gold coupled anti-rabbit secondary antibodies. Bars, 200 nm for A and B, 100 nm for C-F.

*Different cell types have different storage efficiencies* — Secretory granules serve as storage compartments for hormones. In Fig. 21 we determined intracellular storage in different fibroblast cell types. SgII was used as a regulated cargo protein because it is naturally sulfated, and it produced granule-like structures in all transfected cells. A C-terminally tagged version of A1Pi with the tyrosine sulfation sequence SAEDYEYPS (Leitinger *et al.*, 1994) was used as a control for a constitutively secreted protein. Transfected COS-1 and HeLa cells were pulse-labeled with [ $^{35}$ S]sulfate for 60 min at 37°C, followed by a chase for up to 6 h. In parallel, we analyzed stable HEK293 T-REx cells expressing SgII. Because [ $^{35}$ S]sulfate labeling did not work in HEK cells, we pulse-labeled with [ $^{35}$ S]methionine for 30 min at 37°C, followed by a 6 h chase. After the chase time, cells and media were immunoprecipitated, separated by gel electrophoresis and analyzed.

In COS-1 cells, 41% of SgII was retained after a 6 h chase while 94% of A1Pi was already secreted after 2 h. In HeLa cells, even 72% of SgII was retained after 6 h while ~90% of A1Pi was secreted already after 2 h. For SgII, the signal after the chase time (C+M) was partially lost in comparison to total (T) which is an indication for partial degradation. Curiously only ~4% of SgII was stored in stable HEK cells. We do not know for what reasons intracellular storage varied between different cell types.

We also tested if SgII could influence intracellular storage of pro-vasopressin in a stable cell system where SgII expression could be regulated. However, storage in HEK293 cells proved to be generally poor, and we could not observe a cross-effect of SgII expression on intracellular retention of pro-vasopressin (data not shown). The cellular fraction of pro-vasopressin was not complex glycosylated (Endo H sensitive) and was possibly retained in the ER.

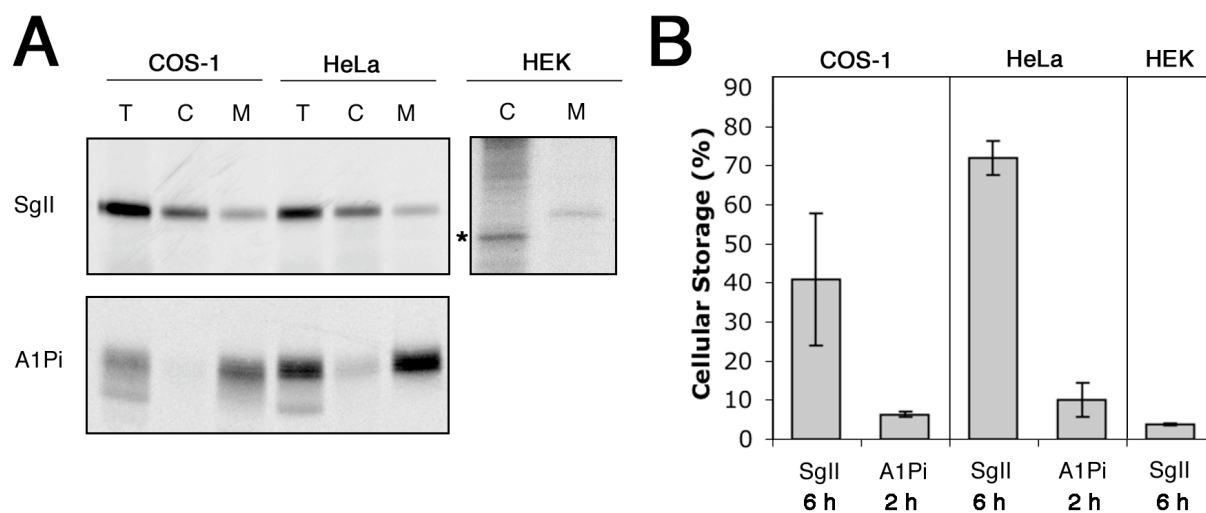


FIG. 21. Intracellular storage of SgII varies between cell types.

A: COS-1 and HeLa cells transfected with SgII-C1 or A1Pi<sup>TS</sup> were pulse-labeled with [ $^{35}$ S]sulfate and chased for 2 or 6 h, as indicated. Stable HEK293 cells expressing SgII-myc were pulse-labeled with [ $^{35}$ S]methionine and chased for 6 h. Cells (C) and media (M) were collected and analyzed by SDS-PAGE. Pulse-labeled protein without chase (T) was analyzed in parallel. B: Representative experiments were quantified and presented in a bar graph. C+M represent 100%. The asterisk (\*) indicates a background band.

*Prohormone convertases require additional machinery for efficient sorting into secretory granules* — Prohormone convertase (PCs) are endoproteases involved in processing and activation of various precursor proteins (Seidah and Chretien, 1999). The endocrine-specific family members PC2, PC3, and PC6A function in the TGN and in the lumen of immature secretory granules. Do PCs have similar characteristics as prohormones and granins to coaggregate with regulated cargo? Or can they even trigger the formation of an aggregate themselves? This can be tested by expression in non-endocrine cells, either alone or together with a granin for example, and analyzed for the formation of granule-like structures or cosorting with the granin, respectively.

In transiently transfected COS-1 cells, only PC3 (Fig. 22A, a) was observed in granule-like structures in around 43% of expressing cells, while PC2 (Fig. 23A, a), PC6A (Fig. 22B, a), and PC7 (Fig. 23B, a) did not lead to the formation of granule-like structures. Similar observations were made in stable cell lines. Stable HEK293 T-REx cells expressing PC3 (Fig. 22A, a) showed strong staining of the Golgi and a small number of granule-like structures around the Golgi region. Stable expression of PC2 (Fig. 23A, e), PC6A (Fig. 22B, e), and PC7 (Fig. 23B, e) in HEK293 T-REx cells resulted in reticular and Golgi staining.

PC3 was able to induce granule-like structures, however, in markedly less efficient way than granins. The other tested members of the PC family were not observed in granule-like structures. PCs may thus require additional machinery or the help of aggregating proteins, such as granins, for segregation into granule-like structures. To test the requirement for granins we cotransfected COS-1 cells with SgII together with either PC2, PC3, PC6A, or PC7. As expected, SgII was observed in the Golgi and in granule-like structures throughout all cells. PC3 and PC6A colocalized with some SgII structures (Fig. 22A and B, b-d), however, many SgII structures were devoid of PC3 and PC6A. Cosorting was not as efficient as when SgII assisted sorting of pro-vasopressin which resulted in colocalization in virtually all granule-like structures (Fig. 19). PC2 and PC7 were not detected in granule-like structures induced by SgII (Fig. 23A and B, b-d). These data were confirmed in stable HEK293 T-REx cells in which PC2, PC3, PC6A or PC7 was permanently expressed and SgII under the control of a tetracycline-inducible promoter. When SgII expression was induced for 24 h, it was detected in granule-like structures throughout the cells. PC3 and PC6A colocalized with SgII in a fraction of the granule-like structures (Fig. 22A and B, f-h), while PC2 and PC7 did not colocalize with SgII in granule-like structures (Fig. 23A and B, f-h).

In summary, PC3 and PC6A were inefficiently sorted into granule-like structures induced by SgII. It is possible that both proteins have affinity to SgII and therefore coaggregate. In contrast, PC2 and PC7 were not sorted into SgII structures at all. This suggests that efficient sorting requires additional machinery which may be exclusive to endocrine cells.



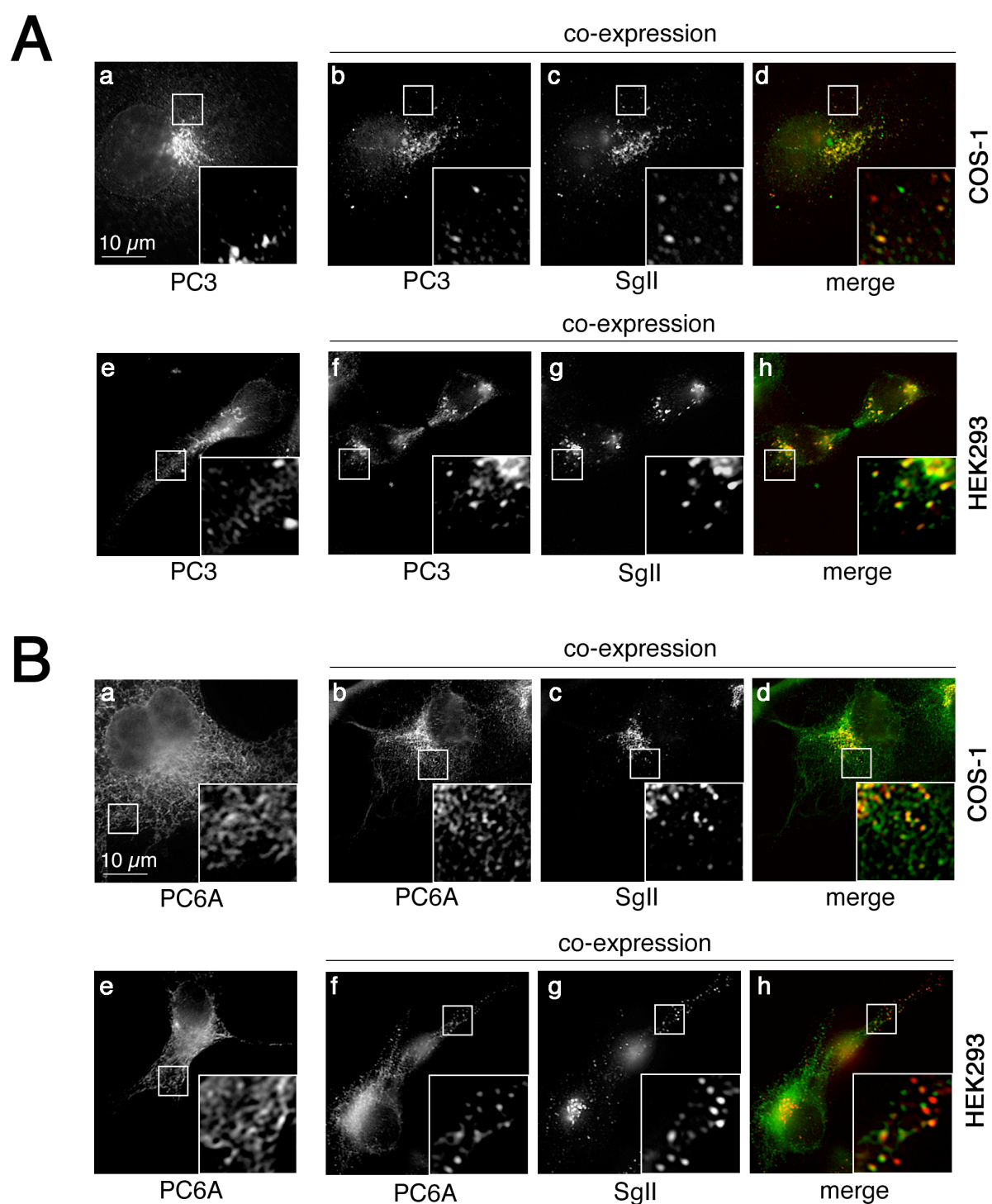


FIG. 22. PC3 and PC6A colocalize with SgII in a large fraction of granule-like structures.

A: PC3-C1 was either expressed alone (a and e) or together with SgII-myc (b-d and f-h). Transiently transfected COS-1 cells (a-d) and stable inducible HEK293 T-REx cells (e-h) were stained with antibodies against the C1-epitope (green) and the myc-epitope (red). B: PC6A-C1 was either expressed alone (a and e) or together with SgII-myc (b-d and f-h). Transiently transfected COS-1 cells (a-d) and stable inducible HEK293 T-REx cells (e-h) were stained with antibodies against the C1-epitope (green) and the myc-epitope (red).

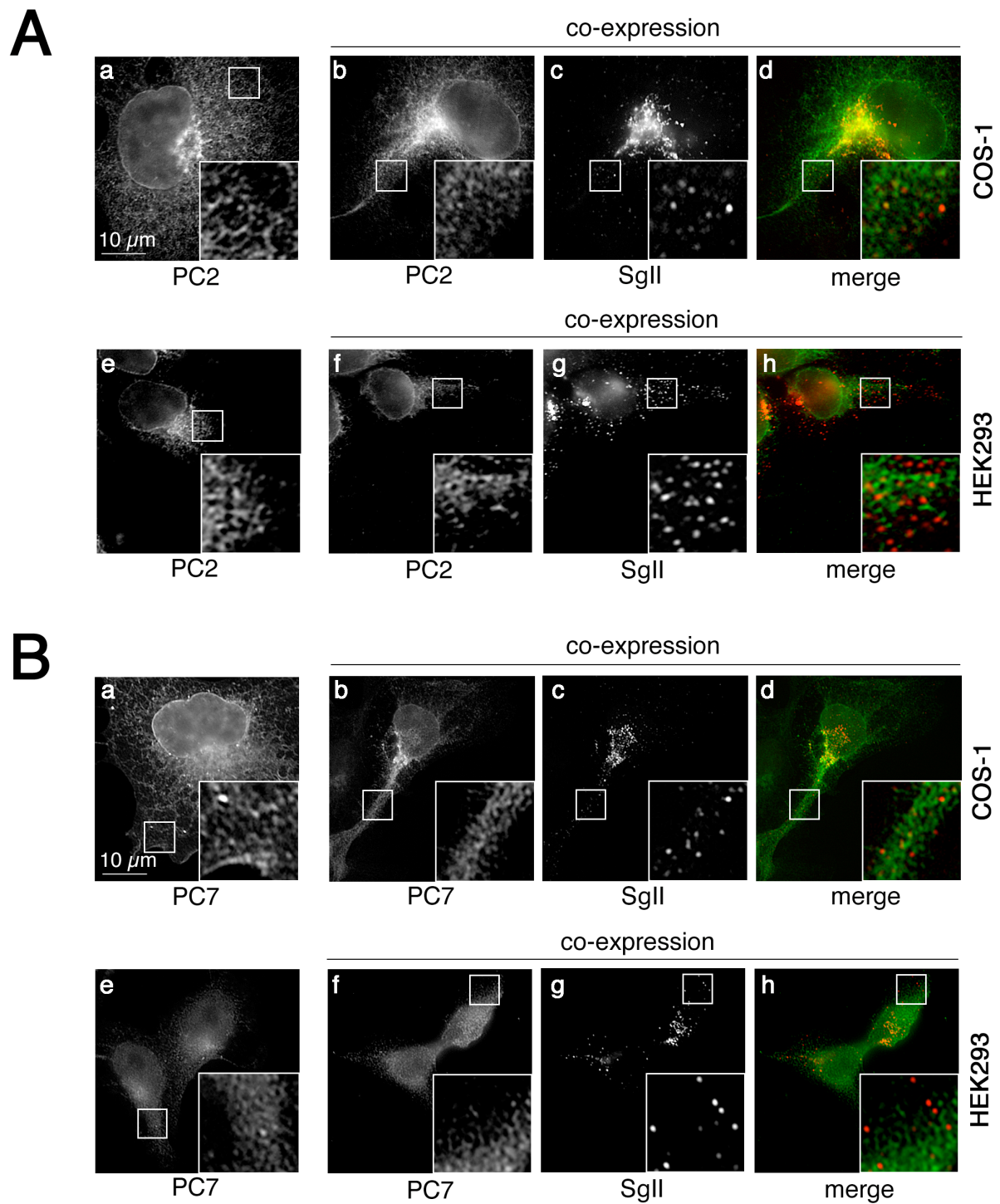
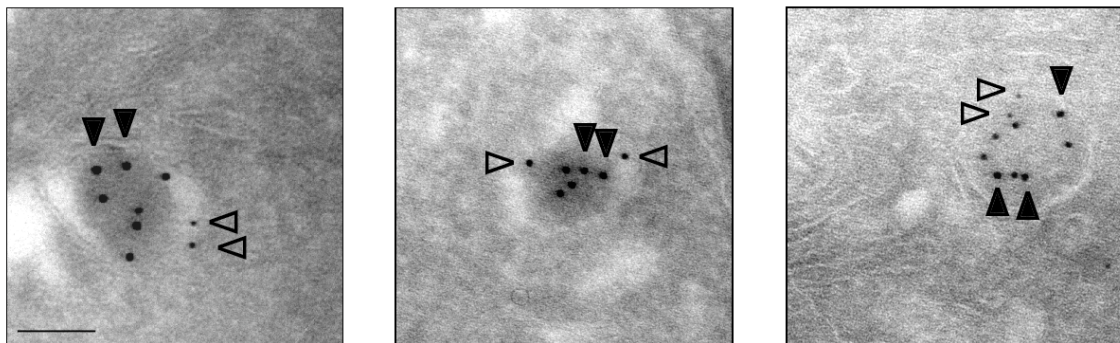


FIG. 23. **PC2 and PC7 do not colocalize with SgII in granule-like structures.**

A: PC2-C1 was either expressed alone (a and e) or together with SgII-myc (b-d and f-h). Transiently transfected COS-1 cells (a-d) and stable inducible HEK293 T-REx cells (e-h) were stained with antibodies against the C1-epitope (green) and the myc-epitope (red). B: PC7-C1 was either expressed alone (a and e) or together with SgII-myc (b-d and f-h). Transiently transfected COS-1 cells (a-d) and stable inducible HEK293 T-REx cells (e-h) were stained with antibodies against the C1-epitope (green) and the myc-epitope (red).

*The IP3-Receptor is recruited to the membranes of SgII granule-like structures* — The IP3-R/ $\text{Ca}^{2+}$  channel, involved in  $\text{Ca}^{2+}$  mobilization from the ER, was shown to be enriched in secretory granules of NIH3T3 cells induced by CgA and CgB to similar levels as in secretory granules of neuroendocrine cells (Huh *et al.*, 2005). Therefore, secretory granules were postulated to play a role as an intracellular  $\text{Ca}^{2+}$  storage organelle with granins acting as calcium ion absorbents. CgA and CgB were shown to directly interact with IP3-R and to activate the inositol 1,4,5-trisphosphate receptor/ $\text{Ca}^{2+}$  channel (Yoo *et al.*, 2002). CgA, CgB, and SgII belong to the granin family and have a high  $\text{Ca}^{2+}$  binding capacity. It was therefore of interest to see if IP3-R is recruited to the membranes of granule-like structures induced by SgII. IP3-R could possibly be recruited by direct interaction of its luminal part with SgII, or alternatively, it enters secretory granules by default due to its abundance in TGN membranes. Cryosections of HEK S18 expressing SgII were decorated with immunogold labeled antibodies directed against secretoneurin and IP3-R. The electron micrographs in Fig. 24 show that SgII was detected in the lumen of electron-dense structures (dark zones) while IP3-R was detected on the surrounding membranes. This is consistent with the function of IP3-R as a membrane  $\text{Ca}^{2+}$  channel, and the results are in agreement with the findings of Huh *et al.* (2005). Besides, the IP3-R is the first example of a protein of neuroendocrine secretory granules that was shown to be recruited to the membranes of granule-like structures.



**FIG. 24. SgII and IP3-R localize to granule-like structures.**

HEK S18 cells expressing SgII-myc under the control of a tetracycline-responsive promoter were induced for 24 h. Cells were stained with primary antibodies directed against the myc-epitope and the IP3-R and secondary antibodies conjugated to 10-nm (SgII, filled arrowheads) and 5-nm (IP3-R, empty arrowheads) gold particles. Bar, 100 nm.



## Discussion

Granule-like structures are induced in non-endocrine cells when a regulated secretory protein is expressed. We observed that only a fraction of the cells expressing pro-vasopressin, pro-oxytocin, or POMC generated granule-like structures, and in the case of pro-vasopressin the structures were considerably bigger than for other cargo proteins. The granins SgII and CgB induced granule-like structures in all transfected cells. In general, we observed that granins had a stronger granulogenic effect than prohormones. Interestingly, when SgII was coexpressed with pro-vasopressin, the number of structures containing a prohormone was increased and their morphology changed to the small type usually observed for cells expressing granins alone. In addition, we found that SgII expression in the background of a stable cell line expressing pro-vasopressin leads to a higher number of granule-like structures containing pro-vasopressin per cell. These structures were positive for both expressed cargo proteins as shown by immunofluorescence (Fig. 19) and electron microscopy (Fig. 20). The efficiency of granule formation was clearly increased and newly generated structures received both cargo proteins. These observations suggest that SgII plays a helper role in generating granule-like structures and in sorting other cargo proteins into them.

When stable V55S34 cells expressing pro-vasopressin were induced for expression of SgII for 24 h some granule-like structures still only contained pro-vasopressin. Even after a prolonged incubation time of 11 days (data not shown) some structures (<1%) were devoid of SgII. This can be explained by observations made by Solimena and Gerdes (2003) where secretory granules were tagged with a DsRed dye that changed color over time. The authors found that newly generated secretory granules are preferentially exocytosed while older ones remain in the cells for prolonged times. The pro-vasopressin-positive granule-like structures observed after more than a day of induction could therefore represent old structures that stayed in the cell for a long time.

Electron microscopy showed that granule-like structures are formed at the TGN. Upon SgII induction pro-vasopressin cosorted with the granin and newly generated granule-like structures could therefore be easily distinguished from old structures. In our system, granule-like structures are not positive for Lamp-1 arguing against granule-like structure forming in the degradative pathway to lysosomes. However, pro-vasopressin and SgII were occasionally detected in multivesicular bodies, suggesting that in the absence of an efficient secretion mechanism (e.g. missing transport and plasma membrane docking mechanism, and SNAREs) granule-like structures may also be eliminated by autophagy.

We also investigated the sorting mechanism of accessory proteins. Sorting of the proprotein convertases PC3 and PC6A into granule-like structures was facilitated by SgII, however, cosorting was not as efficient as observed for pro-vasopressin and SgII. Yet PC2 was not sorted into granule-like structures at all. Its mechanism for sorting appears to require other endocrine-specific proteins. 7B2 was shown to be required for efficient transport, folding, and activation of PC2 (Benjannet *et al.*, 1995; Zhu and Lindberg, 1995). 7B2 might be such a factor which is required for efficient sorting of PC2 into SgII-containing granule-like structures. PC7 is special in that it is a transmembrane protein with a cytoplasmic domain. It is

believed to traffic between TGN and plasma membrane, similar to furin, and it is usually not found in mature secretory granules. However, furin is incorporated into immature secretory granule from where it is recycled to the TGN prior to vesicle maturation via CCVs (Dittie *et al.*, 1997), and this may also be the case for PC7. In transiently transfected COS-1 cells, PC7 expression showed reticular staining and was also observed at the cell surface (Fig. 23B, a) which is an indication for cell surface trafficking of PC7. PC2, PC3, and PC6A are luminal proteins, and it has been shown that an amphipathic  $\alpha$ -helix close to the C-terminus is required for membrane interaction and sorting of these enzymes. However, our data suggest that there must be other mechanisms for PC sorting than membrane interaction, aggregation or coaggregation with secretory cargo proteins.

We observed that intracellular storage of secretory proteins varied between non-endocrine cell types. After a 6 h chase, SgII storage was ~40% in COS-1 and even more than 70% in HeLa cells while virtually no storage was observed in HEK293 cells. Our previous results showed a lower storage efficiency in COS-1 cells where only 20% of pulse-labeled CgB, and 45% of SgII (25% when labeling was performed at 37°C) was retained after a chase time of 6 h (Beuret *et al.*, 2004). When CgB was stably expressed in MDCK cells, ~20% of pulse-labeled CgB was retained after a 2 h chase (Wacker *et al.*, 1997). Pulse-chase experiments to analyze the intracellular retention of CgA revealed that ~20% was stored in CV-1 cells after a 3 h chase (Malosio *et al.*, 2004). Although in PC12 cells 99% of labeled endogenous CgA was retained in the cells after 3 h, a significant amount of transfected CgA was stored in CV-1 cells. Interestingly, only ~70% of vaccinia-expressed CgB was sorted in PC12 secretory granules after a 90 min chase (Kromer *et al.*, 1998). A possible explanation for the different storage efficiencies observed between cell types could be explained by variations in TGN conditions. The most obvious conditions known to be important for aggregation of secretory granule cargo are an acidic pH and high  $\text{Ca}^{2+}$  concentrations. The luminal pH of organelles in the regulated secretory pathway becomes more acidic in the progress of the pathway ( $\text{pH}_{\text{ER}}$  7.2-7.5,  $\text{pH}_{\text{Golgi}}$  6.2-6.6,  $\text{pH}_{\text{SG}}$  5.0-5.5) (Machen *et al.*, 2003). Similarly, calcium ion concentrations increase from 3 mM in the ER to 10 mM in the TGN to 20-50 mM in the lumen of secretory granules (Chanat and Huttner, 1991). The pH in the TGN was determined to be 6.38 in HEK293 cells (Machen *et al.*, 2003), 6.58 in HeLa cells (Llopis *et al.*, 1998), and 6.17 in human skin fibroblasts (Seksek *et al.*, 1995) (Vero 6.45, CHO 6.25). In neuroendocrine cells, however, the values are similar with a pH 6.2 in the Golgi of AtT-20 cells (Wu *et al.*, 2001). Considering the small variations between  $\text{pH}_{\text{TGN}}$  values and the rather average value for HEK293 cells, it seems unlikely that differences in TGN acidity could account for the low storage efficiency of HEK293 cells.

Our results indicate that the initial steps of secretory granule formation are based on a self-assembly process. However, sorting of accessory proteins, efficient storage, transport, and fusion require mechanisms that are not present in fibroblasts.

## CHAPTER III

### **Proprotein Convertase PC3 Is Not a Transmembrane Protein**

Biochemistry, 2005, Volume 44, 5339-5345

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## Abstract

Proprotein convertase PC3 (also known as PC1) is an endopeptidase involved in proteolytic processing of peptide hormone precursors in granules of the regulated secretory pathway of endocrine cells. Lacking any extended hydrophobic segments, PC3 was considered to be a secretory protein only peripherally attached to the granule membrane. Recently, evidence has been presented that PC3 is a transmembrane protein with a 115-residue cytoplasmic domain and a membrane-spanning segment containing eight charged amino acids [Arnaoutova, I., *et al.* (2003) *Biochemistry* 42, 10445-10455]. Here, we analyzed the membrane topology of PC3 and of a PC3 construct containing a conventional transmembrane segment of 19 leucines. Alkaline extraction was performed to assess membrane integration. Exposure to the cytosol or to the ER lumen was tested by addition of C-terminal tags for phosphorylation or glycosylation, respectively. Protease sensitivity was assayed in permeabilized cells. The results show that the C-terminus of PC3 is translocated across the endoplasmic reticulum membrane. Furthermore, the proposed transmembrane segment of PC3 and a similar one of carboxypeptidase E did not stop polypeptide translocation when inserted into a stop-transfer tester construct. PC3 is thus not a transmembrane protein. These results have implications for the mechanism of granule sorting of PC3 as well as for the topology of PC2 and carboxypeptidase E, which have been reported to span the lipid membrane by homologous charged sequences.

## Introduction

Intracellular processing and maturation of prohormones and proneuropeptides is achieved by common proteolytic mechanisms. The processing enzymes include a family of endopeptidases, the proprotein convertases (PCs), that cleave hormone and neuropeptide precursors at dibasic sites (Steiner, 1998; Zhou *et al.*, 1999; Thomas, 2002), and a family of metallo-carboxypeptidases that selectively remove the basic amino acids exposed at the new C-termini (Fricker, 1988b). PCs belong to the kexin/subtilisin family of serine proteases. Eight mammalian PCs have been identified. They share a common organization, illustrated in Fig. 26A for PC3 (also known as PC1 or PC1/3), with an N-terminal signal peptide for targeting to the endoplasmic reticulum (ER), a propeptide, a catalytic domain, a P-domain, and a variable C-terminal segment. In the case of PC3, the prodomain is autocatalytically cleaved already in the ER, converting the ~95-kDa full-length precursor to a ~85 kDa intermediate. In secretory granules, a further autoproteolytic cleavage occurs at the C-terminus of the P-domain, producing a ~64-kDa form with full enzymatic activity.

Some members of the PCs (furin, PC6/6B, and PC7) contain an obvious transmembrane segment in their C-terminal domain and are thus type I membrane proteins. The other members of the family (PC1/3, PC2, PC4, PC5/6A, and PACE4) were generally assumed to be fully translocated into the ER lumen as soluble or peripherally membrane-associated proteins. Indeed, in vitro-translated PC2 and PC3 were shown to associate with TGN/secretory granule membranes and lipid rafts (Blazquez *et al.*, 2000; Blazquez *et al.*, 2001). In addition, PC2 and PC3 contain a C-terminal sequence potentially forming an amphipathic helix associating to membranes peripherally. However, evidence has recently been presented based on protease protection, antibody binding, and chemical modification experiments with purified secretory granules, suggesting that PC3 is in fact a type I transmembrane protein with a cytoplasmic C-terminus (Arnaoutova *et al.*, 2003b) (Fig. 25). The sequence proposed to act as the membrane-spanning segment was identified as residues 619–638, directly adjacent to the P-domain (QNDRRGVEKMVDPGEEQPTQENPKENTLVS).

Similarly, residues 617–634 at the very end of the 40-amino acid C-terminal domain of PC2 were proposed to function as a transmembrane anchor mainly based on membrane-impermeant biotinylation of purified chromaffin granules (Assadi *et al.*, 2004) (SKLAMSKK-EELEEEELDEAVERSLKSILNKN-COOH).

These sequences were initially identified by similarity to the C-terminal sequence of carboxypeptidase E (CPE), which on isolated secretory granules was found to be sensitive to carboxypeptidase Y digestion and accessible to antibodies against the C-terminus (Dhanvantari *et al.*, 2002) (FELESFSERKEEEKEELMEWWKMMSETLNF-COOH).

The transmembrane topology of these proteins is of particular importance, because these sequences and the potentially cytoplasmic domains are implicated in lipid raft association and sorting into the regulated secretory pathway. CPE was even proposed to function as a sorting receptor for regulated secretion (Cool *et al.*, 1997), although this has remained controversial (Irminger *et al.*, 1997). The C-terminal portion of CPE was shown to interact with ADP-ribosylation factor 6 (ARF6) in yeast two-hybrid assays and pull-down experi-

ments (Arnaoutova *et al.*, 2003a). A fusion protein of the luminal portion of the IL-2 receptor  $\alpha$  subunit and the 25 C-terminal residues of CPE behaved as a raft-associated protein showing ARF6-dependent recycling from the plasma membrane to the TGN (Arnaoutova *et al.*, 2003a), supporting a membrane-spanning topology of the protein.

However, the three proposed transmembrane segments are highly unusual, since they are composed of 40–60% charged residues, predominantly glutamates. Conventional transmembrane sequences consist of 16–25 uncharged, mainly hydrophobic residues. In single-spanning membrane proteins, charged residues are virtually absent in the core of the lipid bilayer (Reithmeier and Deber, 1992; Landolt-Marticorena *et al.*, 1993). In the exceptional case of individual intramembrane charges in transmembrane segments, they may cause ER retention and degradation, unless neutralized by hetero-oligomerization with a complementary partner protein (Cosson *et al.*, 1991).

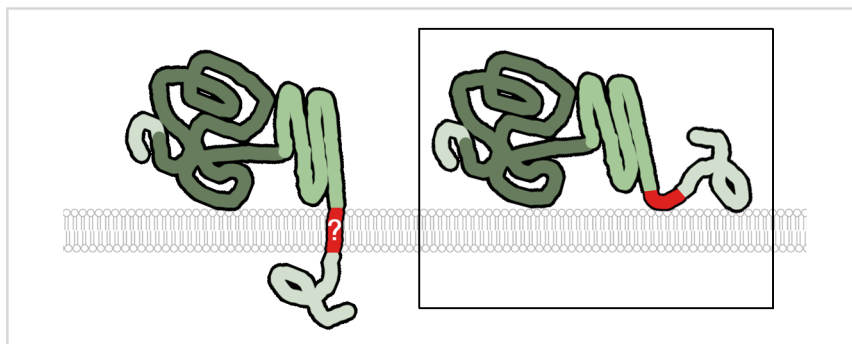


FIG. 25. **Possible topologies of PC3.**

In the left side of the sketch PC3 adopts a transmembrane topology as suggested by Arnaoutova *et al.* (2003b). A fully luminal version of PC3 is shown on the right side. The putative transmembrane domain is depicted in red.

Because of the physiological importance of the question whether these prohormone processing enzymes are membrane-spanning proteins due to a novel type of transmembrane segment, we set out to test the membrane disposition of PC3 using methods developed to analyze membrane protein topology. We chose PC3 as the protein to be investigated, because the proposed membrane-spanning segment is further inside the polypeptide chain. Modifications at the C-terminus of PC3 are 115 amino acids downstream of the proposed transmembrane domain, and it can therefore be excluded that they affect membrane insertion and topology. We also analyzed the proposed transmembrane segments of PC3 and CPE in a stop-transfer tester construct for their ability to stop translocation of a polypeptide across the ER membrane. The results consistently show that these sequences, unlike a hydrophobic Leu<sub>19</sub> sequence, are unable to integrate into the ER membrane and that PC3 is not a transmembrane protein.

## Materials and Methods

*DNA constructs* — The cDNA of human PC3 was a generous gift by Wim van de Ven (Flanders Institute of Biotechnology, Leuven). To insert a classical transmembrane domain of 19 consecutive leucines, a Bgl II site was introduced by polymerase chain reaction (PCR) in front of the sequence encoding the oligoleucine segment of H1ΔQLeu19 (Wahlberg and Spiess, 1997) using the mutagenic primer GCGAGATCTGTTGCTTTTGCTGCTG and fused in-frame to the 5' portion of PC3 up to the BamH I site at codon 625. The 3' portion of PC3 was amplified with the primer CGCAGATCTGGGGAGGAGCAGCCC introducing a Bgl II site before codon 627 and ligated in-frame into the BamH I site at the end of the oligoleucine sequence. In the wild-type PC3 sequence and the oligoleucine insertion mutant, the stop codon was replaced by the sequence of an Asp718 site by PCR using the antisense primer CGCGGTACCAGAATTTTCCTCATTCAGAATGTC and fused via Asp718 to the C-terminal 14 codons of the asialoglycoprotein receptor H1, which encode the C1 epitope recognized by a rabbit anti-peptide antiserum (Beuret *et al.*, 2004).

To introduce a C-terminal glycosylation tag, the C-terminal 40 codons of H1ΔL22[110] (Goder and Spiess, 2003), which encode residues 141-160 of H1 with an N-glycosylation site and the C1 epitope, were amplified with a primer introducing a 5' BsrG I site to be ligated into the Asp718 site at the 3' end of the PC3 or PC3-L19 sequences. Similarly, the sequence of the C1 epitope was amplified with a primer containing a BsrG I site, and the sequence encoding the consensus heptapeptide sequence for phosphorylation by protein kinase A and ligated to the PC3 or PC3-L19 sequences. The resulting C-terminal peptide extensions of PC3 are shown in Fig. 26C.

To generate a size marker for unprocessed PC3, the segment encoding the signal sequence (codons 2–26) was deleted from the cDNA of C1-tagged PC3 by PCR using the primer GCG-AAGCTTACCATGGCGAAAAGGCAATTTGTC (the Hind III site used for subcloning is underlined). cDNA constructs encoding H1 and H1-M (lacking the transmembrane domain) were described previously (Spiess and Lodish, 1986).

The stop transfer tester constructs STT and STT-H1<sup>38–65</sup> were derived from plasmids pSA0 and pSAA described previously (Wessels and Spiess, 1988; Spiess *et al.*, 1989). Codons 599–657 of PC3 and codons 409–476 of CPE were amplified using primers providing a 5' Acc I and a 3' Cla I site for ligation into the Cla I site of pSA0. All constructs were verified by sequencing and inserted into the expression plasmid pECE (Ellis *et al.*, 1986).

*In vivo expression and labeling* — COS-1 cells were grown in Dulbecco's modified Eagle's minimal essential medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin at 37°C with 7.5% CO<sub>2</sub>. Transient transfection was performed with lipofectin (Life Technologies) according to the manufacturer's protocol in 6-well clusters. The cells were processed 2 days after transfection.

Transfected cells were starved for 30 min in methionine-free medium, labeled with 100 µCi/ml [<sup>35</sup>S]methionine in starvation medium for 30 min at 37°C, and washed with cold phosphate-buffered saline (PBS). The cells were lysed and immunoprecipitated using a

rabbit antiserum directed against the C1 epitope. The immune complexes were isolated with protein A-Sepharose, analyzed by SDS-polyacrylamide gel electrophoresis and phosphorimaging. For deglycosylation, immune complexes were incubated in 50 mM K-phosphate, 25 mM EDTA, 2% Triton X-100, 0.2% SDS, 1% 2-mercaptoethanol, in the presence or absence of 0.2 mU or 5 mU endoglycosidase H (for partial or complete deglycosylation, resp.) for 1 h at 37°C.

For labeling with [<sup>32</sup>P]phosphate, cells were starved in phosphate-free medium and labeled for 30 min with 100 µCi/ml g-[<sup>32</sup>P]ATP in the presence of 20 µM forskolin. Cells were lysed in the presence of phosphatase inhibitors (500 µM nitrophenyl phosphate, 50 µM sodium orthovanadate, 1 mM sodium fluoride, 1mM EDTA) and further processed as above.

For Western analysis, cells were boiled in SDS-sample buffer, proteins separated by gel electrophoresis and transferred to polyvinylidene fluoride membrane that was then incubated in blocking buffer (PBS with 0.1% Tween-20 and 5% nonfat dry milk) for 60 min and with anti-C1 antibody in blocking buffer for 60 min at 4°C. Bound antibody was detected using horseradish peroxidase-conjugated anti-rabbit immunoglobulin secondary antibody and the ECL kit (Amersham Biosciences).

*Alkaline extraction and protease protection* — [<sup>35</sup>S]Methionine-labeled cells were extracted under alkaline conditions as described previously (Wessels *et al.*, 1991) by homogenization in Hepes buffer, pH 11.5, for 15 min on ice. One half of each sample was centrifuged through a sucrose cushion, and the membrane pellet, the supernatant, and the untreated sample were immunoprecipitated separately and analyzed. For protease protection, [<sup>35</sup>S]methionine-labeled cells were swollen in 15 mM Hepes/KOH, pH 7.2, 15 mM KCl, scraped, pelleted, and resuspended in 1.2 ml PBS. Aliquots were incubated for 30 min on ice with or without 200 µg/ml trypsin or with trypsin and 1% Triton X-100. Reactions were stopped with 400 µg/ml soybean trypsin inhibitor before immunoprecipitation and analysis by SDS-gel electrophoresis and autoradiography.



## Results

To test membrane insertion of PC3, its sequence was extended by short carboxyterminal diagnostic tags, as shown in Fig. 26A to reveal the disposition of the C-terminus of the protein. PC3-g has a 22-residue extension with an efficient site for N-glycosylation to report exposure of the C-terminus to the ER lumen. In contrast, PC3-p is tagged with a 9-amino acid consensus sequence for phosphorylation by cAMP-dependent protein kinase in the cytosol (Hjelmquist *et al.*, 1974; Kemp *et al.*, 1977; Goder *et al.*, 2000). These two constructs and the wild-type sequence were furthermore provided with a 16-residue C1 epitope for efficient immunoprecipitation. As a further control, a conventional transmembrane domain composed of an uninterrupted stretch of 19 leucine residues (L19) was inserted into the region of the proposed stop-transfer sequence of PC3, generating PC3-L19, PC3-L19g, and PC3-L19p (Fig. 26A and C).

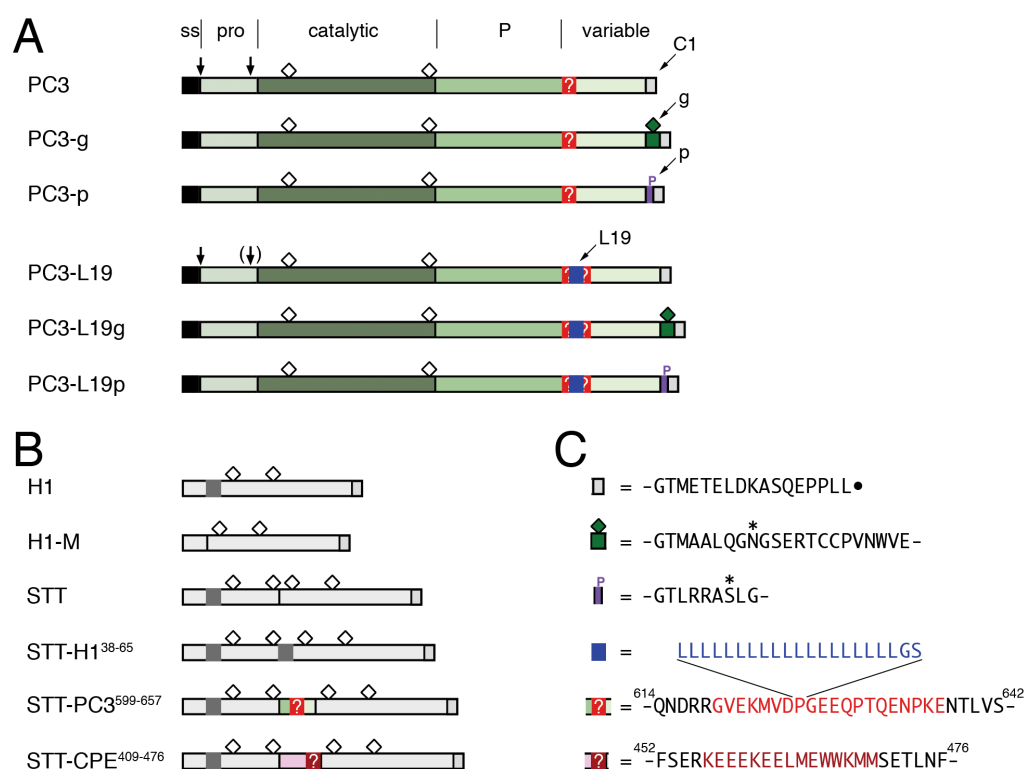
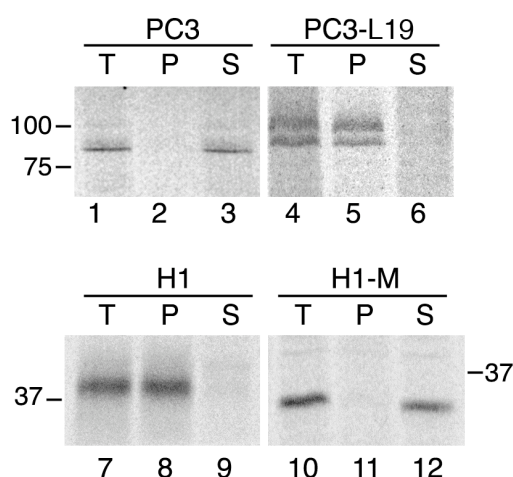


FIG. 26. **PC3 constructs to study membrane topology.**

**A:** The domain organization of PC3 and derived constructs are schematically shown. PC3 consists of a signal sequence (ss), the pro-domain (pro), the catalytic portion, the P-domain, and a C-terminal segment that is variable among the PC family members. The proposed transmembrane segment (residues 619–638) is highlighted in red with a *question mark*. Potential glycosylation sites are indicated by *diamonds*. Vertical arrows indicate cleavage sites (in parenthesis where cleavage is slowed down by insertion of the L19 sequence). The leucine<sub>19</sub> sequence inserted into the proposed transmembrane segment, the C1-epitope tag, the glycosylation, and the phosphorylation tags are indicated by L19, C1, g, and p, respectively. **B:** Schematic representations of the stop-transfer tester construct (STT) and its derivatives containing the signal-anchor of the asialoglycoprotein receptor H1 (H1<sup>38–65</sup>), segment 599–657 of PC3, or 409–476 of CPE are shown. **C:** The amino acid sequences of the relevant elements in the PC3 and STT constructs shown in *panels A and B* are listed. A *dot* represents the C-terminus and *asterisks* the residues modified by glycosylation or phosphorylation.

*PC3 is solubilized upon alkaline extraction* — Under strongly alkaline conditions (pH >11) biological membranes have been shown to be converted to open membrane sheets. Soluble and peripheral membrane proteins are released, whereas integral membrane proteins generally remain embedded in the lipid bilayer and are pelletable (Fujiki *et al.*, 1982). This method proved to be a useful empirical procedure to identify integral membrane proteins.

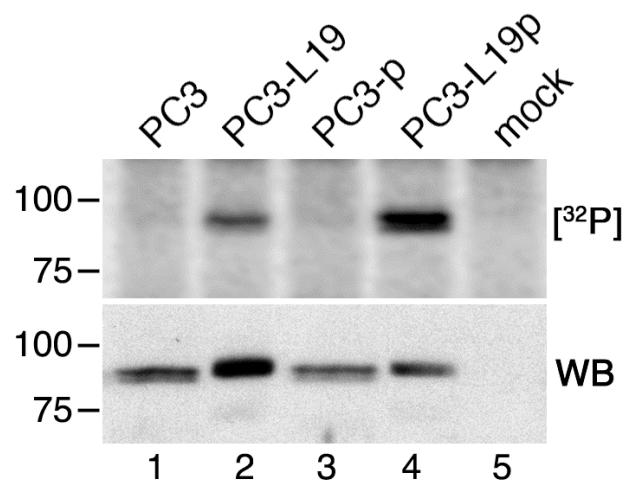
PC3 and PC3-L19 were transiently expressed in COS-1 cells in parallel with the asialoglycoprotein receptor H1, a single-spanning type II membrane protein, and H1-M, the same protein lacking its transmembrane segment, as controls. Cells were labeled with [<sup>35</sup>S]methionine for 30 min and then extracted at pH 11.5. Extracted material and membranes were separated by centrifugation through a sucrose cushion. Total material, pellet, and supernatant were analyzed by immunoprecipitation, SDS-gel electrophoresis, and autoradiography (Fig. 27). As expected, H1 was recovered exclusively in the membrane pellet (lanes 7–9) and H1-M in the soluble fraction (lanes 10–12). PC3 was produced as a single species of the expected apparent molecular weight of ~85 kDa, corresponding to the protein lacking its N-terminal pro-domain (lane 1). It was efficiently extracted into the supernatant (lanes 1–3). Expression of PC3-L19 generated two products differing in size by ~8 kDa (lane 4). The two species correspond to the pro-form and the N-terminally processed form. The presence of the oligoleucine sequence appears to slow down autocatalytic cleavage of the pro-domain. Both forms of PC3-L19 remained completely membrane associated due to the oligoleucine sequence (lanes 4–6). The proposed transmembrane segment of PC3 thus cannot anchor the protein in the lipid bilayer under alkaline conditions. However, while alkaline extraction as an empirical method provides quite reliable results for conventional membrane proteins, the proposed PC3 transmembrane segment rich in glutamate and aspartate under harsh deprotonating conditions may follow different rules.



**FIG. 27. PC3 is released from the membrane upon alkaline extraction.**

Transfected COS-1 cells expressing PC3, PC3-L19, H1, or H1-M were labeled with [<sup>35</sup>S]methionine for 30 min and subjected to alkaline extraction and centrifugation to separate soluble and membrane integrated proteins. The total before centrifugation (T), and the supernatant (S) and membrane pellet (P) after centrifugation were analyzed by immunoprecipitation, SDS-gel electrophoresis, and autoradiography. The positions of marker proteins with their molecular weights in kDa are indicated.

*The C-terminus of PC3 is not accessible to cytosolic protein kinase* — To test for the cytosolic exposure of the C-terminus, the PC3 construct with a C-terminal phosphorylation site (PC3-p), wild-type PC3, and the corresponding oligoleucine-containing constructs (PC3-L19p and PC3-L19) were expressed in COS-1 cells, labeled for 30 min with [ $^{32}$ P]phosphate, and analyzed by immunoprecipitation against the C1 epitope, SDS-gel electrophoresis, and autoradiography. As a control, transfected cells were analyzed by Western blotting to reveal steady-state levels of PC3 constructs in the cells. PC3 and PC3-p were not detectably labeled with [ $^{32}$ P]phosphate (Fig. 28, lanes 1 and 3). In contrast, PC3-L19p was strongly phosphorylated (lane 4), supporting a transmembrane disposition of the protein with the oligoleucine segment as a transmembrane domain. Even PC3-L19 without the phosphorylation tag was labeled (lane 2), indicating that the C-terminal region of PC3, which is rich in serines and threonines, is unspecifically phosphorylated when exposed to the cytosol. These results thus indicate that the C-terminus of PC3 is not exposed to the cytosol in intact cells.

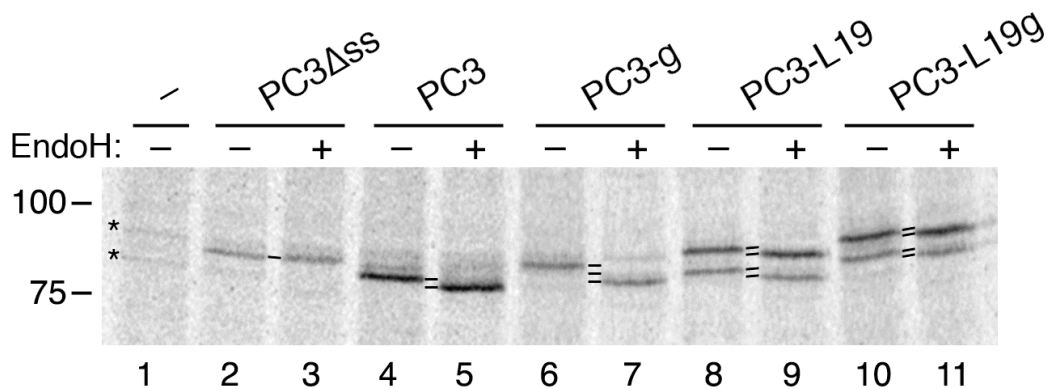


**FIG. 28. PC3 is not phosphorylated at a C-terminal phosphorylation site.**

Transfected COS-1 cells expressing PC3, PC3-L19, or the corresponding constructs PC3-p, or PC3-L19p with a C-terminal site for phosphorylation by protein kinase A in the cytosol, were either labeled for 30 min with [ $^{32}$ P]phosphate ([ $^{32}$ P]) and analyzed by immunoprecipitation, gel electrophoresis, and autoradiography, or subjected to Western blot analysis (WB) as a measure of steady-state expression levels. In parallel, mock-transfected cells were analyzed. Since generally the steady-state levels of PC3 and PC3-p were lower than those of the corresponding L19 constructs, three times more sample was loaded in lanes 1 and 3. The positions of marker proteins with their molecular weights in kDa are indicated.

*The C-terminus of PC3 is exposed to the ER lumen* — N-Linked glycosylation is a luminal ER modification frequently used to assay protein topology, since it provides positive evidence for luminal localization of glycosylation sites. Human PC3 contains two potential sites for N-glycosylation, both within the catalytic domain. Upon expression in COS-1 cells and labeling for 30 min with [ $^{35}$ S]methionine, a major species of the expected apparent molecular weight of ~85 kDa was produced (Fig. 29, lane 4). This form corresponds to the processed protein lacking the 83-residue pro-domain, which is autocatalytically removed. Deglycosylation of the protein by endoglycosidase H digestion results in a shift of ~3 kDa (lane 5), indicating that PC3 is glycosylated at a single site. As a size marker, a truncation construct lacking the signal sequence (PC3 $\Delta$ ss) was expressed in parallel (lanes 2 and 3). The resulting uncleaved and unglycosylated pro-PC3, was larger than the deglycosylated PC3 product by ~8 kDa consistent with the size of the pro-domain.

PC3-g, due to the additional glycosylation tag, showed an electrophoretic mobility like PC3 $\Delta$ ss (Fig. 29, lane 6). Deglycosylation generated twice the mobility shift observed for PC3 (lane 7), indicating that PC3-g was glycosylated twice: in the catalytic domain and in the C-terminal extension. The C-terminus of PC3 is thus exposed to oligosaccharyl transferase in the ER lumen, contradicting a transmembrane topology. Expression of PC3-L19 and PC3-L19g generated two products each, with and without the pro-domain (Fig. 29, lane 8 and 10). Upon deglycosylation, all forms shifted by only ~3 kDa, indicating a single glycosylation. The additional glycosylation site at the C-terminus of PC3-L19g was therefore not translocated into the ER lumen, consistent with a type I membrane-spanning topology.



**FIG. 29. The C-terminus of PC3 is exposed to the ER lumen.**

Transfected COS-1 cells expressing PC3, PC3-L19, or the corresponding constructs PC3-g, or PC3-L19g with an additional site of N-glycosylation at the C-terminus were labeled with [ $^{35}$ S]methionine for 30 min and subjected to immunoprecipitation. The immunoprecipitates were split in half and incubated with (+) or without (-) endoglycosidase H (EndoH) before gel electrophoresis and autoradiography. In parallel, untransfected cells (-) were analyzed and cells transfected with PC3 $\Delta$ ss lacking the signal sequence, as a size marker. Asterisks indicate background bands. Mobility shifts due to deglycosylation are indicated by lines. The positions of marker proteins with their molecular weights in kDa are indicated.

*PC3 is protected from exogenous protease by the ER membrane* — An alternative method to assay for cytosolic protein domains is protease protection. Using isolated secretory granules, the 86 kDa form of PC3 was previously found to be sensitive to trypsin digestion (Arnaoutova *et al.*, 2003b). We applied this method to PC3 while still in the ER. Transfected COS-1 cells were labeled for 30 min with [<sup>35</sup>S]methionine, swollen, and scraped to break the plasma membrane. The permeabilized cells were then incubated with or without trypsin, or with trypsin and detergent before immunoprecipitation and analysis. H1, which was analyzed as a control, was resistant to trypsin digestion except for its N-terminal cytoplasmic domain of 40 amino acids (Fig. 30, lanes 7–9). PC3 was completely protected from hydrolysis, except when the membrane was dissolved with detergent (lanes 1–3). In contrast, PC3-L19 disappeared upon trypsin treatment, indicating that the C-terminal epitope was exposed on the outside of the microsomal membranes. The results support the conclusion that PC3 resides entirely within the ER lumen.

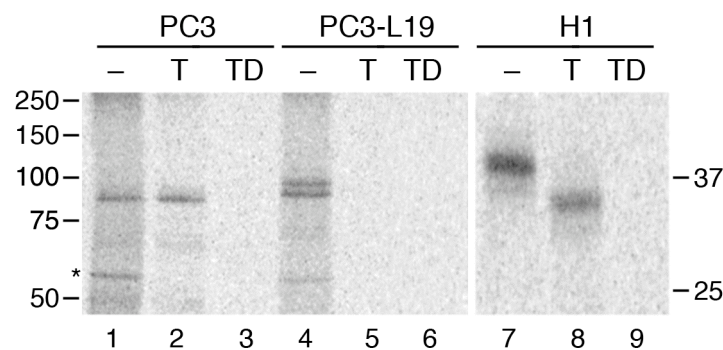


FIG. 30. **The C-terminus of PC3 is protected from protease digestion by the ER membrane.**

Transfected COS-1 cells expressing PC3, PC3-L19, or the control protein H1 were labeled with [<sup>35</sup>S]methionine for 30 min, broken by swelling and scraping and incubated on ice with or without trypsin (T) or detergent (D). After addition of trypsin inhibitor, samples were lysed, immunoprecipitated, and analyzed by gel electrophoresis and autoradiography. An unspecific background band is indicated by an asterisk. The positions of marker proteins with their molecular weights in kDa are indicated.

*The proposed transmembrane segments of PC3 and CPE do not function as stop-transfer sequences* — We previously created a stop-transfer tester construct, STT, on the basis of sequences of H1 (Fig. 26B) (Wessels and Spiess, 1988; Spiess *et al.*, 1989). It consists of the N-terminal cytosolic domain, the internal signal-anchor sequence, and a partially duplicated C-terminal domain of H1 with 4 sites for N-linked glycosylation. The signal-anchor targets the protein to the ER and induces translocation of the C-terminal domain. Upon expression in COS-1 cells and [<sup>35</sup>S]methionine-labeling, STT is found as a major species with four glycans and a minor one with three (Fig. 31, lanes 1–4). This is verified by partial deglycosylation with endoglycosidase H, yielding a ladder of intermediates corresponding to polypeptides with 0–4 N-glycans. Sequences inserted between the second and the third glycosylation site can be tested for their ability to stop further translocation of the polypeptide. If they do, like in the



case of a second copy of the signal-anchor of H1 in STT-H1<sup>38-65</sup> (lanes 5–8), maximally two glycosylation sites will reach the ER lumen. As observed previously (Wessels and Spiess, 1988), the resulting protein is glycosylated only once, because the second site is too close to the second transmembrane segment.

In the construct STT-PC3<sup>599-657</sup>, the proposed transmembrane domain of PC3 together with approximately 20 flanking residues on both sides was inserted into STT. When expressed in COS-1 cells, the products were glycosylated three- and fourfold (Fig. 31, lanes 9–12), just like STT without insertion. Similarly, the C-terminal 68 residues of CPE, including the segment of residues 457–471 that had been implicated to span the membrane, produced three- and fourfold glycosylation (lanes 13–16). The results indicate that both sequences cannot stop protein transfer in the ER. In summary, the results demonstrate that PC3 is not a type I membrane protein and that the proposed transmembrane segments of PC3 and CPE are not functional as stop-transfer sequences in the ER.

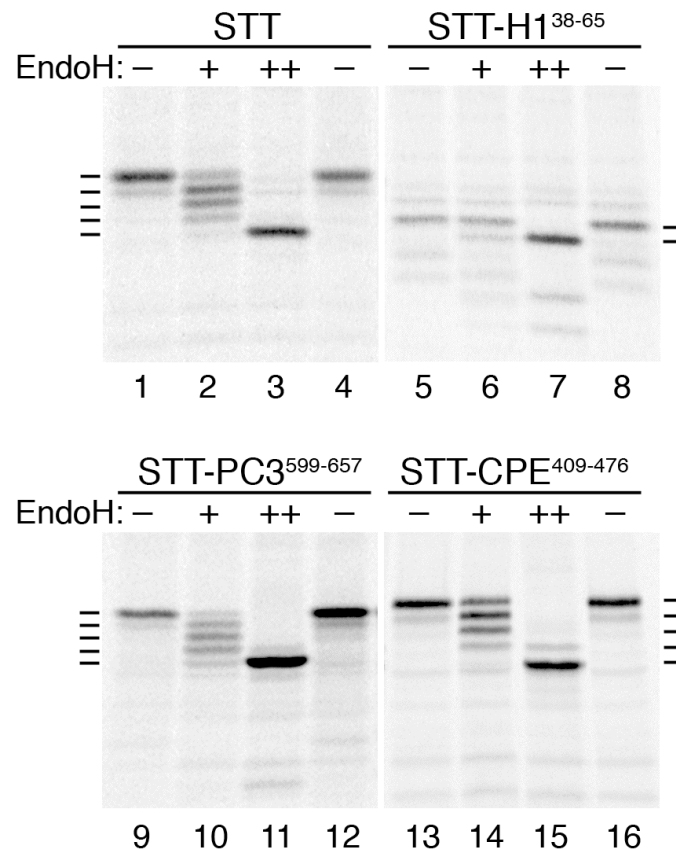


FIG. 31. The proposed transmembrane segments of PC3 and CPE do not stop polypeptide translocation in the ER.

Transfected COS-1 cells expressing the stop-transfer tester (STT) constructs without insertion, with the signal-anchor sequence of H1, with residues 599–657 of PC3, or residues 409–470 of CPE were labeled with [<sup>35</sup>S]methionine for 30 min and subjected to immunoprecipitation. Aliquots of the immunoprecipitates were incubated with 0 (–), 0.2 mU (+), or 5 mU (++) of endoglycosidase H (EndoH), and analyzed by gel electrophoresis and autoradiography.

## Discussion

Because PC2, PC3, and CPE lack a conventional hydrophobic segment in their sequence, they were initially considered to belong to the class of secretory proteins. All three proteins were found, however, to associate with membranes, particularly with lipid rafts, a feature that was proposed to mediate their sorting into secretory granules (Fricker, 1988a; Blazquez *et al.*, 2000; Dhanvantari and Loh, 2000; Blazquez *et al.*, 2001). Membrane association thus qualified them for classification as peripheral membrane proteins. The proposal that these three proteins are integral membrane proteins spanning the lipid bilayer (Dhanvantari *et al.*, 2002; Arnaoutova *et al.*, 2003b; Assadi *et al.*, 2004) challenged this conventional model as well as the traditional concepts on the properties of transmembrane segments.

Secretion of PC2, PC3, or CPE activity into the medium is mainly attributed to forms cleaved at the end of the P-domain, releasing functional protein without its putative membrane anchor. In the case of PC3, however, secretion of a 85-kDa form that includes its C-terminal portion has been observed in the medium of cultured cells (Zhou *et al.*, 1995; Boudreault *et al.*, 1998). This indicated that at least a fraction of PC3 is not spanning the membrane. This leaves the possibility of partial anchoring of the polypeptide by an inefficient stop-transfer sequence. An example where partial transmembrane insertion was observed is the prion protein (Hegde *et al.*, 1998; Hegde and Rane, 2003). It is normally synthesized with an N-terminal cleaved signal and a C-terminal glycosyl phosphatidylinositol attachment sequence as a translocated form called <sup>Sec</sup>PrP. A fraction of the proteins, however, inserts into the bilayer via an internal segment to produce the membrane-spanning forms <sup>Ntm</sup>PrP and <sup>Ctm</sup>PrP. The transmembrane segment is mildly hydrophobic, which explains its behavior as an "inefficient" stop-transfer sequence in a conventional way. In artificial proteins, the minimal hydrophobicity to stop polypeptide translocation was found to correspond to a stretch of 19 alanines or 9 leucines (Kuroiwa *et al.*, 1991).

The proposed transmembrane domains of PC2, PC3, and CPE violate the rule that polypeptides crossing the hydrophobic core of the membrane have to be predominantly apolar. As an explanation, it has been put forward that at acidic pH, as in the TGN or secretory granules, glutamic and aspartic acid residues are partially protonated, and that within the lipid bilayer the  $pK_a$  may dramatically increase (Dhanvantari *et al.*, 2002). Yet, the energy for increasing the  $pK_a$  by one pH unit calculated according to the formula  $\Delta G^\circ = RT \cdot 2.303 \cdot \Delta pK_a$  amounts to  $\sim 5.7$  kJ/mol. This may be compensated by the transfer of neighboring apolar residues into the hydrophobic environment of the bilayer. In the proposed transmembrane segments, potentially charged residues outnumber hydrophobic residues by far. In addition, even for the transfer of protonated acidic side chains into an apolar environment, the energy for removing hydration water must be accounted for. Energetically, insertion of the proposed sequences into the bilayer thus appears highly unfavorable.

In the case of PC3, spontaneous membrane insertion in an acidic organelle can be excluded, because the translocation of 115 residues would have to be accomplished. For this reason, it was suggested that the transmembrane orientation of PC3 is established during synthesis as the protein enters the rough ER (Arnaoutova *et al.*, 2003b). The proposed transmembrane sequence, upon interacting with the ER membrane, would stop further translocation of the

remainder of the C-terminal domain, which would stay on the cytosolic side of the membrane. This is what we have experimentally tested in the present study. We have used COS-1 cells as a convenient cell system. The machinery for membrane integration in the ER is unlikely to differ between cell types. Indeed, PC3 and PC3-g expressed in Neuro2a cells, a neuroendocrine cell line, behaved exactly as in COS-1 cells (shown in the supplementary material, Fig. 32). In our experiments, we found PC3 to be released from membranes upon alkaline extraction (Fig. 27), an empirical method to distinguish integral membrane proteins from peripheral or secretory proteins. We could not detect any phosphorylation of a C-terminal target sequence of protein kinase A (Fig. 28), but efficient glycosylation of a C-terminally attached glycosylation site (Fig. 29). The protein was furthermore resistant to protease digestion in permeabilized cells (Fig. 30). The proposed transmembrane segment and its flanking sequences also scored negative as part of a construct designed to test for stop-transfer activity (Fig. 31). In all tests, PC3 behaved as expected for a completely translocated protein, whereas PC3 with an inserted Leu<sub>19</sub> sequence showed all the hallmarks of a type I integral membrane protein. The results lead to the conclusion that neither segment 619–638 nor any other sequence in PC3 spans the lipid bilayer in a significant fraction of the molecules.

The simplest explanation to rationalize the discrepancies between our experiments and those described by Arnaoutova *et al.* (Arnaoutova *et al.*, 2003b) is contamination of secretory granules by PC3, in particular the 85-kDa form, from broken ER or Golgi during granule purification. This material might be peripherally bound to the outside of the granule membrane and account for antibody binding, biotinylation, and protease sensitivity. In contrast, the phosphorylation and glycosylation assays used here reflect the situation in intact, living cells. For protease protection, the cells' plasma membrane was broken by swelling and scraping without further treatment before incubation with or without trypsin.

The conclusion that PC3 is not a membrane-spanning protein has implications on potential sorting mechanisms of the protein, since direct interaction with cytosolic factors can be excluded. Interaction with lipid rafts and/or raft-associated components is limited to the exoplasmic surface of the TGN and granule membranes. With respect to the proposed transmembrane segment of CPE, our stop-transfer test shows that it is not capable to halt translocation of a polypeptide chain in the ER. Spontaneous integration in a later compartment of the secretory pathway cannot be excluded from the data, but the energetics of membrane integration do not make it seem likely. For polypeptide segments crossing the lipid membrane, there is no convincing alternative to hydrophobic sequences, certainly not in single-spanning proteins.

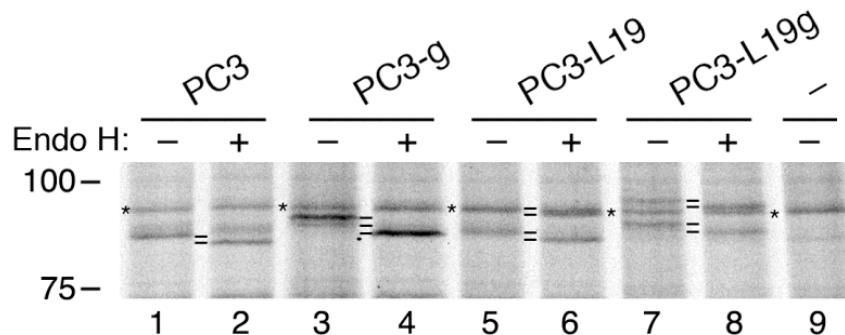


## Supplementary Material

Endocrine cells might have a different system for integrating polypeptides into the lipid bilayer than fibroblast cells. All experiments in our publication (Stettler *et al.*, 2005) were performed in COS-1 cells since they provide a convenient cell system for studying protein topology. To rule out the possibility that PC3 behaves differently in specialized cells, the glycosylation assay (Fig. 29) was repeated in Neuro2a cells (Fig. 32).

Upon expression of PC3 Neuro2a and labeling with [<sup>35</sup>S]methionine a band was observed at the expected apparent molecular weight of ~85 kDa (Fig. 32, lane 1) representing PC3 without prodomain. Endoglycosidase H treatment resulted in a size shift of ~3kDa (lane 2), indicating that PC3 was glycosylated at a single site, most likely at one of the two potential glycosylation sites within the catalytic domain. PC3-g was larger than PC3 due to its additional glycosylation tag (lane 3). Upon treatment with endoglycosidase H, a mobility shift of ~6 kDa was observed (lane 4) indicating that PC3-g is glycosylated twice, at an internal site and at the C-terminus. The C-terminus is therefore exposed to the ER lumen and accessible to the oligosaccharyl transferase as previously observed in COS-1 cells (Fig. 29). PC3-L19 and PC3-L19g were glycosylated at a single site only (lanes 6-9) indicating that the C-terminal glycosylation site of PC3-L19g was not translocated into the ER lumen, as expected for a type I membrane-spanning protein.

In summary, PC3 and PC3-g behaved in Neuro2a exactly as in COS-1 cells. Therefore, it can be ruled out that PC3 adopts a different topology in (neuro)endocrine cells than in non-endocrine cells.



**FIG. 32. The C-terminus of PC3 is exposed to the ER lumen.**

Transfected Neuro2a cells expressing PC3, PC3-L19, or the corresponding constructs PC3-g, or PC3-L19g with an additional site of N-glycosylation at the C-terminus were labeled with [<sup>35</sup>S]methionine for 30 min and subjected to immunoprecipitation. The immunoprecipitates were split in half and incubated with (+) or without (-) endoglycosidase H (EndoH) before gel electrophoresis and autoradiography. In parallel, untransfected cells were analyzed. Asterisks indicate background bands. Mobility shifts due to deglycosylation are indicated by lines. The positions of marker proteins with their molecular weights in kDa are indicated.



## GENERAL CONCLUSIONS

The formation of secretory granules has long been considered an endocrine-specific process. Although it is not clear what the molecular players for segregation of regulated secretory cargo and the subsequent formation of secretory granules are, it has generally been accepted that the necessary machinery is exclusively present in specialized cells. Interestingly, when we expressed regulated secretory proteins in fibroblast cell lines, we observed discrete structures in the cell periphery. Regulated cargo proteins that were able to induce granule-like structures include prohormones (pro-vasopressin, pro-oxytocin, and POMC), granins (CgB and SgII) and the prohormone convertase PC3. Among these tested proteins, granins were most efficient. Furthermore, granins played a supportive effect in sorting hormones into granule-like structures and had an influence on the granule morphology when coexpressed. These findings clearly underline the helper role of granins. In ongoing studies, we have analyzed the sorting of CgA into granule-like structures. Interestingly, N-terminal truncation mutants of CgA that were constitutively secreted from neuroendocrine PC12 cells (Taupenot *et al.*, 2002) did not induce granule-like structures in COS-1 cells, and they could not be redirected into granule-like structures when SgII was coexpressed. These data indicate that the protein segments required for sorting into the regulated pathway of PC12 cells are important for self-aggregation and co-aggregation with SgII.

When SgII was expressed in COS-1 cells, the IP3-R/ $\text{Ca}^{2+}$  channel was sorted into granule-like. The IP3-R/ $\text{Ca}^{2+}$  channel could possibly be recruited by direct interaction of its luminal domain with SgII, as it was shown to be the case for CgA and CgB (Yoo *et al.*, 2002), or it enters by default due to its abundance in TGN membranes. IP3-R is the first protein of neuroendocrine secretory granules which was shown to be recruited to granule-like structures. However, IP3-R is not exclusively localized on secretory granules, but it is also found in the ER and the nucleus. Nevertheless, these findings imply that accessory proteins have affinity to granins even if they are not capable of inducing granule-like structures themselves. Granins may build heteroaggregates with other regulated secretory cargo and escort them into granule-like structures acting as a guide. Yet, it is not clear if pro-vasopressin can also recruit the IP3-R into granule-like structures. Further experiments are under way.

Live-imaging of SgII-DsRed transfected COS-1 cells stimulated with the ionophore A23187 resulted in an undirected movement of granule-like structures in the cell (data not shown). The structures did not move towards the plasma membrane nor was any fusion event observed within 15 min after stimulation. Granule-like structures did not move in a directed way but rather appeared to move back and forth staying more or less in place. It appeared that granule-like structures do not have the necessary machinery for movement to the plasma membrane and for fusion. It remains unclear if and how these structures do fuse with the plasma membrane.

Video microscopic studies on CgB-GFP transiently expressed in Vero cells revealed intervals of rapid directed movement of transport carriers leaving the TGN (Wacker *et al.*, 1997). Transport was shown to occur in a microtubule-dependent manner, but movement was discontinuous and occurred in all directions sometimes even changing directions. For direct

and fast transport to the plasma membrane secretory granules are dependent on endocrine-specific machinery.

In the following section, the similarities and differences between granule-like structures and real secretory granules of specialized cells are summarized.

Criteria underlining that granule-like structures *resemble* secretory granules:

- Proteins are highly concentrated as concluded from immunofluorescence signals.
- Dense cores were observed by electron and differential interference contrast microscopy as well as by density gradient centrifugation (not shown).
- Electron microscopic analysis showed that membrane-bounded structures form at the TGN.
- Immunofluorescence microscopy showed that granule-like structures do not co-localize with markers for endosomes and lysosomes.
- Coexpressed cargo proteins sorted together into granule-like structures.
- Cargo proteins were segregated from a constitutively secreted marker protein.
- Biochemical storage of regulated secretory proteins was observed in pulse-chase experiments.
- Stimulated release of regulated secretory proteins was triggered by ionophore.
- Granins played a helper role in sorting prohormones and influenced the morphology of granule-like structures.
- Prohormone convertases PC3 and PC6A sorted into granule-like structures when coexpressed with SgII.
- The IP3-R/ $\text{Ca}^{2+}$  channel was detected on the membranes of granule-like structures.
- Sorting of CgA mutants into PC12 secretory granules correlates with the formation of granule-like structures in COS-1.

Criteria demonstrating that granule-like structures *differ* from secretory granules:

- Granule-like structures are larger than secretory granules.
- Precursor proteins are not processed because the appropriate enzymes are not expressed.
- Prohormone convertase PC2 was not sorted into granule-like structures when coexpressed with SgII.
- Intracellular storage varied from hardly detectable levels for pro-vasopressin to above 70% for SgII.
- Storage in HEK293 cells was very inefficient.
- Stimulated secretion was inefficient and slow.

- Live-imaging showed that granule-like structures were not efficiently transported to the cell periphery.
- Fusion with the plasma membrane was not observed in stimulated cells.

Non-endocrine cells provide a convenient system to study the role and effects of individual secretory proteins in isolation. In the future, additional factors (e.g. proteins involved in transport, docking and fusion) could be tested for their potency to enhance the functionality of granule-like structures.



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